THE ROLE OF IMMUNOGLOBULIN E (IgE) IN RESPONSE TO NEOPLASIA

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A thesis submitted to the Faculty, Department of Internal Medicine, Yale University, in partial fulfillment of the requirements for the degree, Doctor of Medicine

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TABLE OF CONTENTS

LIST OF TABLES AND FIGURES

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List of Abbreviations Used

PCA -passive cutaneous anaphylaxis

ACA -active cutaneous anaphylaxis

P-K -Prausnitz-Kustner

PBS -Phosphate buffered saline

i.v.-intravenous

i.p.-intraperitoneal

s.c.-subcutaneous

i.d.-intradermal

Ig -immunoglobulin

OA -ovalbumin

RIA -radioimmunoassay

SRS-A -slow reacting substance of anaphylaxis

CBH -cutaneous basophil hypersensitivity

AMP -adenosine monophosphate

ECF -eosinophilic chemotactic factor

SRBC -sheep red blood cells

H&E -Hematoxylin and Eosin

PHA -Passive hemagluttination

BGG -Bovine gamma globulin

PREFACE

As a first year medical student I naively wondered, what is the relationship between allergy and cancer? After all, I reasoned with a limitless lack of sophistication, the immune system must have something to do with both diseases. When I posed this question two years later to John Dwyer, my adviser, I received the answer that stimulated much of what I now report. His reply was, "The question has merit. Go to the library. Find out what you can. Report back to me." Dutifully I began to search the journals trying to resolve such broad issues as do atopics develop cancer as often as normals?; could those with allergy have a genetic selective advantage in immunosurveillance, the rejection of malignant clones before their clinical presentation?; what is the role of IgE, that immunoglobulin class primarily responsible for allergy, in responding to neoplasia? Armed with questions like these, a pattern was established. I would read and report; John Dwyer would listen, question, suggest. Slowly we elaborated projects to resolve specific issues.

The laboratory in which these experiments were undertaken possesses many skills in immunological research. In particular, the lab is expert in assessing T and B cell function and investigating cutaneous basophil hypersensitivity responses. The laboratory's lack of prior experience with research on IgE provided me with great latitude in experimentation, but this also meant that a substantial amount of time had to be devoted to basics before the proposed tumor work could be successfully engineered.

The thesis then contains three natural divisions. Part I stems directly from that first naive question; it is a review of the literature regarding the relation between IgE and neoplasia. Part II derives from the need to understand some basic properties of IgE. It describes studies that have no direct relation to tumor immunology but includes such fundamental topics as the assay of IgE and the ontogenetics of IgE production. Finally, Part III concerns specifically the relation between IgE and tumors.

In many ways, this work is unfinished. This is not the study's defect, but rather its virtue. The topics explored were constantly demanding clarification of results, suggesting further study, and requiring refinement of techniques. This thesis project resembles the process of education by representing the construction of a starting gate from which to conduct a marathon, not a dash. While the finish line has not yet been reached, many hurdles have been cleared and most important, a direction in which the runners can head has been established.

The naive medical student has changed little since his initial question, but he has discovered that even questions asked out of ignorance may merit exploration.

PART I: A Review of the Literature

Few topics in medicine are currently generating as much interest as the relationship between the immune system and malignancy. Recent reviews[192,208,245] have covered such relevant issues as blocking antibodies, cell-mediated immunity, tumor associated antigens, immunosurveillance, immunodeficiency, and immunotherapy. With such a plethora of interests demonstrating a definite relationship between the immune system and cancer, not surprisingly all reviews omit any mention of the ambiguous role that immunoglobulin E or IgE plays in response to neoplasia. Yet much suggests the importance of just this subject.

Background

Basic facts about IgE, which is sometimes called reaginic, skin-sensitizing, or homocytotropic antibody, have been reviewed thoroughly and the reader is referred elsewhere[24,92] for detailed information. In brief, as recently as ten years ago, the antibody responsible for immediate wheal and flare skin reactions was thought to be a subclass of IgA. Then, in 1966, careful work by K. Ishizaka and his colleagues showed reaginic antibody to be a unique class of immunoglobulin.[93,94] This was confirmed shortly thereafter by the discovery of an IgE myeloma,[104] and the demonstration that antisera to the myeloma protein could absorb reaginic activity from serum.[254] Several parasitic infections[171,196,210] and atopic diseases[210] including asthma,[103,121] allergic rhinitis,[134] and atopic eczema[106] are often characterized by elevated levels of IgE. Reagin is thought to be primarily responsible for immediate type hypersenstivity responses such as anaphylaxis. Some evidence suggests that IgE might have a role in such diverse entities as Graves' disease[239] and the nephrotic syndrome.[70,133] IgE is present in the serum in ex-

tremely low concentrations,[105] nanograms per milliliter, and is also tissue bound to basophils and mast cells.[15,99] An IgE-antigen reaction can trigger the release of potent mediators including histamine,[255] slow reacting substance of anaphylaxis (SRS-A)[255], and eosinophilic chemotactic factor.[110]

Intuitive Arguments

The idea that individuals with atopy may have a significantly lower incidence of malignancy has occurred to many investigators. One intuitive argument could be stated as a syllogism: Cancer begins as a failure of the immune system, an immunodeficiency; in allergy, the immune system is over-active or hypersensitive; therefore, perhaps the immunosurveillance system of an atopic is more likely to reject a clone of malignant cells before a cancer is clinically apparent.

In rejecting this logic, a response might stress that the pathogenesis of atopy should not be considered merely as an excess of IgE or an exaggeration of the normal immune response. First, no study has shown that all those with asthma, allergic rhinitis, or atopic eczema, have increased total IgE; roughly thirty to sixty per cent of atopics can be characterized as such,[34,105,106,121,134,210] although an elevation of antigen-specific IgE antibodies does correlate more closely with the incidence of allergic symptoms.[134] Second, atopics clearly have other immune aberrations. Recent studies have suggested that T-cell function, both as tested in vivo by delayed hypersensitivity skin testing and in vitro as tested by mitogen stimulation, is depressed in atopic disease,[75,138,155,262] although studies in our lab and elsewhere have not confirmed these findings.[38,52,55,65] Serafini has shown

that allergic patients have markedly increased autoantibodies of the thyro-gastric group.[203] Evidence suggests that reaginic antibodies are qualitatively and not just quantitatively abnormal in atopic persons.[251] Some have contended that the primary immune disturbance in allergic individuals is actually a transient IgA deficiency, not an IgE elevation.[217,226] And numerous excellent studies in animals are demonstrating the role of the T-cell in regulating IgE.[79,81,174-176, 222-225]

A second intuitive argument considers genetic laws. The incidence of atopy may be as high as twenty-four percent of the population.[207] Allergy has long been recognized as familial[45] and now evidence has mounted that both in[20,21,130,150] man and mouse[151,229,230] IgE levels are genetically determined. One would hope to find then some selective advantage in being allergic, but none has been proven. While IgE levels are clearly elevated in many parasitic infestations, only debatable arguments claim any benefit from this elevation.[34,171, 125,126] Some have suggested that IgE protects against respiratory infections if IgA is absent[7,87] and that possibly the IgE system serves as a warning about pollutants, but equally good data indicate that IgE in the presence of IgA deficiency predisposes to pulmonary disease.[184] Individuals apparently perfectly normal have been shown to be IgE deficient.[132,184] Although without substantiation, the argument is tantalizing. If a quarter of the population has a genetic defect, namely the tendency to develop allergic symptoms, then there must be an unrecognized selective advantage somehow involved with this "defect".

Hoping to show that this selective advantage involves the response to tumors, at least nine separate investigations have been published on

the incidence of allergy or atopy in patients with malignancies.[56,63,66,139,141,156,159,204,228] The first such study[139] published in 1953 actually concluded that patients with cancer, mainly carcinoma of the cervix in this report, were far more likely to have allergies (27%) as compared to controls (11%). The study has several major defects, however, including the authors' effort to prove an hypothesis that they had formulated before the investigation, thus biasing their inquiries, and the lack of age or sex matched controls.

Of the eight subsequent studies along this line, five concluded that patients with malignancy had a significantly decreased incidence of atopy, while three studies disclosed no difference between normals and patients with cancer. These studies all differ markedly in such essential parameters as the definition of allergy or atopy, the care in matching controls, the range in types of malignancy studied, and the size of the population. Some of the findings of all nine studies are summarized in Table I. The publication of so many reports has served to confuse rather than clarify, as evidence accumulates that atopy is both relevant and irrelevant to the incidence of carcinoma.

Total Serum IgE and Malignancy

With the ability to measure serum IgE, however, it seemed that the controversy could be settled more definitively by simple assaying IgE in the serum of normals and those with cancer. Indeed, at least four groups of investigators have tried this approach, but here, too, opinion is not unanimous.

Augustin and Chandradasa[13] were first to publish their results on serum IgE in patients with neoplasia in 1971. Using a radial immunodiffusion technique to measure IgE, they found that the mean serum value

TABLE I STUDIES ON ATOPY AND CANCER INCIDENCE

AUTHOR & DATE	RANGE OF ATOPY OR ALLERGY	NUMBER OF SUBJECTS	DESCRIPTION OF SUBJECTS	% CANCER PTS ATOPY	% CONTROL ATOPY	COMMENTS
LOGAN & SAKER 1953	ASTHMA, HAY FEVER, ECZEMA	CANCER -195	MOSTLY CERVICAL CHRCINOMA	27	11	ONLY STUDIED TO CONCLUDE THAT ALLERGICS HAVE INCREASED INCIDENCE OF MALIGNANCY
DWORIN ET AL 1955	HAY FEVER, ASTHMA, URTICARIA ECZEMA, ANGIONEUROTIC EDEMA	TOTAL -200	HODGKIN'S DISEASE ONLY	13	Not Done	NO CONTROLS BUT CONCLUDED THAT INCIDENCE OF MAJOR ALLERGY WOULD PROBABLY HAVE BEEN SIMILAR
FISHERMAN 1960	BRONCHIAL ASTHMA SEASONAL RHINITIS ECZEMA	CANCER 1185 CONTROL 294	PATIENTS PRESSNTING TO CLINICS: WIDE RANGE OF TUMORS INCLUDED	3.2	12.9	MANY ATOPICS HAD REMISSION OF THEIR SYMPTOMS BEFORE CANCERS DEVELOPED; UTERUS, PROSTATE, & BREAST HAD ESPECIALLY LOW INCIDENCE OF ATOPY
MACKAY 1967	ASTHMA, HAY FEVER, NETTLE RASH, ECZEMA	CANCER 150 CONTROL 150	LEUKEMICS EXCLUDED	9.3	20.7	FEMALES WITH TUMORS WERE ESPECIALLY UNLIKELY TO BE ALLERGIC
URE 1969	NASAL ALLERGY	TOTAL 140	ALL PATIENTS ON A GYNECOLOGY WARD	0	20	ALLERGY AND MALIGNANCY WERE MUTUALLY EXCLUSIVE IN THIS SMALL STUDY OF PATIENTS ON A GYNECOLOGY WARD
SHAPIRO ET AL 1971	HAY FEVER, SKIN PROBLEMS, INJECTION REACTIONS	CANCER 605 CONTROL 3310	PATIENTS FROM 3 BOSTON HOSPITALS	7.1	6.6	ATOPY DETERMINED BY NURSE'S ADMISSION DATA AND THE FIRST FOUR DISCHARGE DIAGNOSES
GABRIEL ET AL 1972	ASTHMA, HAY FEVER, ECZEMA, URTICARIA, FOOD REACTION	CANCER 150 CONTROL 150	ALL LUNG CANCERS, CONTROLS WERE OUTPATIENTS	2	14	CONTROLS AGE MATCHED WITH SIMILAR SMOKING INCIDENCE AS CANCER GROUP
McKEE ET AL 1967	SEASONAL RHINITIS OR ASTHMA	TOTAL 403 CANCER 104 CONTROL 299	PATIENT WERE PREOPERATIVE WITH A VARIETY OF TUMORS	13.4	15	ALL SUBJECTS WERE INTERVIEWED PRIOR TO SURGERY SO THAT NEITHER PATIENT NOR INTERVIEWER KNEW IF THE DIAGNOSIS WAS CANCER
MEERS 1973	ASTHMA OR HAY FEVER	CANCER 546 CONTROL 533	PATIENTS UNDER GOING RADIOTHERAPY	4.9	15	SUGGEST THAT IgE MAY SUPPRESS A MALIGNANCY VIA " VASCULOCELLULAR DAMAGE"

of this immunoglobulin was depressed in 65 patients with cancer as compared to 50 controls. But IgE levels were also decreased in 48 hospitalized surgical patients. No statistically significant difference was found between the cancer group and the surgery controls or between the surgery patients and the normals.[263] Still, this report is often cited as one that shows decreased IgE in patients with cancer.

In 1972, Jacobs et al.[102] measured IgE levels in 50 patients with allergy, 100 normals, 200 with untreated advanced carcinoma, and 200 with carcinoma undergoing chemotherapy with cyclophosphamide. Both a radial immunodiffusion and solid phase radio-immunoassay (RIA) were used. Most of the tumors were bronchogenic carcinomas. While the majority of cancer paitents had low levels of IgE, many of the untreated subjects had falsely elevated serum levels as shown by the high levels by RIA and undetectable values by immunodiffusion. This false elevation may be due to an auto-antibody which is also present in normal sera and is of unknown significance.[160] Other authors have also found autoantibodies against IgE in normal serum.[1,242]

Arbesman et al.[9] utilized their own radio-radial immunodiffusion technique to evaluate IgE levels in patients with various diseases including malignancy. Of 282 patients with cancers, 32 had what the authors considered to be an elevated level, greater than 300 units per milliliter. Like the two previous studies, they conclude that IgE seems to be decreased with malignancy, but no statistically significant difference was proven between the cancer group and the blood bank donor controls.

The study of Waldmann et al.[231] was most recently published. The main advantage of their work is the use of a double antibody radio-

immunoassay[72] which is particularly sensitive when low values of IgE or non-specific interference with the assay are possibilities.[185] Their data is also submitted to statistical analysis and includes only immunoglobulin levels of patients on first admission to the hospital, thereby eliminating the complication of therapy possibly altering IgE levels. Studying 331 cases of neoplasia, the authors conclude that IgE levels are generally normal with cancer. However, patients with B-cell malignancies such as multiple myeloma and chronic lymphatic leukemia do have depressed levels of serum IgE just as they have depression of other immunoglobulin classes.[231] This is often explained as a crowding out of the normal B-cell or antibody-producing precursors by neoplastic cells. Finally, and most intriguingly, patients with Hodgkin's disease have clearly elevated levels of IgE which the authors suggest may be due to a depression of T-cell function. Arbesman et al.[9] had likewise shown elevated levels correlating with the stage of the disease.

Numerous investigators are actively examining the role of the T-cell in regulating IgE responses. Most manipulations that destroy T-cell function including thymectomy,[175] irradiation,[173,175] and treatment with anti-lymphocyte serum[240] seem to favor IgE production. One group has isolated a soluble factor from thymocytes that can suppress an IgE response.[223] Since Hodgkin's disease has decreased T-cell function with the incidence of anergy correlating with the stage of the disease, a possible cause of the elevated IgE, especially in stage III and IV disease, becomes apparent.[198]

Although the association may just be coincidental, Hodgkin's disease is associated with two phenomena also related to IgE. The neph-

rotic syndrome not infrequently accompanies Hodgkin's disease, and as IgE may be a causative factor in some forms of nephrosis, at least one physician has speculated that the nephrotic syndrome in Hodgkin's might be related to an IgE response.[41] Second, fifteen to twenty percent of Hodgkin's disease patients have eosinophilia,[56] some to a very marked degree,[250,258] and the speculative possibility exists that this is related to reagin.

Hodgkin's Disease in an Animal Model

The SJL mouse strain develops a spontaneous neoplasm often considered to be an animal model of Hodgkin's disease.[3] Paradoxically, in published reports so far, this mouse has been shown to be a very poor producer of reagin[131] and it has even been noted that its failure to make IgE might relate to its tendency to develop tumors.[129] Despite its seeming inability to produce IgE, the SJL mouse has been reported to be among the most susceptible mice to anaphylaxis,[227] although mice have reaginic antibody other than IgE and non-reaginic factors may also mediate anaphylaxis.

Haran-Ghera et al.[82] have made a thorough study of the SJL mouse in order to consider the possibility of predicting by immune parameters which individuals would develop the spontaneous reticulum cell neoplasm resembling Hodgkin's. Examining such criteria as presence of antinuclear antibodies, antibody response to sheep red blood cells, and rejection of skin grafts, they were unable to distinguish those animals that were to develop tumors. Warren[235] has shown that SJL mice make a poor immune response to schistosoma mansoni eggs when compared to DBA/2 controls. His criterion for response was the size of granulomata in the lung six-

teen days after the eggs were given intravenously, As many believe that IgE is a crucial factor in responses to parasites, a poor reaginic response might account for the smaller granuloma in SJLs.

With the evidence that humans with Hodgkin's disease have increased IgE but that animals that supposedly develop a Hodgkin's-like tumor have low IgE levels, further study is needed. What, for example, are the IgE levels in the mouse after the onset of tumor? Could variability in making IgE have predictive value as to which animal will develop the neoplasia? These studies would be facilitated by the ability to measure total serum IgE in the mouse. This can be done in the rat[22,42] thanks to the discovery of IgE myeloma proteins in this species,[23] but comparable tumors have not been described in the mouse. At present mouse IgE levels can be titered only in relation to a specifice antigen by the method of passive cutaneous anaphylaxis. (See methods in Part II.)

Tumor-Specific IgE Responses

Since nine studies have failed to show conclusively if atopics have a decreased incidence of carcinoma, and four studies on serum IgE seem now to point to normal levels of IgE in malignancies except in Hodgkin's disease (for which a few authors would even question its status as a neoplasia)[4], can IgE still be playing a significant role in response to neoplasia? Some experimental work published in 1958 strongly suggests that IgE may play a critical role in responding to some tumors.

Seventeen years ago, Grace and Kondo[74] reported on eight patients, give with breast cancer, two with Hodgkin's and one with a lymphoma, all of whom had "heat, edema, and tenderness" locally about their tumor, With extracts of each tumor, they produced a wheal and flare reaction within fifteen to twenty minutes after an intradermal injection into the patient's

own skin, an immediate hypersensitivity response as is mediated by IgE. As controls, patients had no reactions to saline or normal tissue. As added proof, the reaction could be passively transferred by first injecting a normal volunteer with appropriate serum intradermally and one day later challenging that site with solubilized tumor antigen. This classic Prausnitz-Kustner (P-K) reaction following a twenty-four hour sensitization must be mediated primarily by IgE,[91,186,211] but as IgE was not known to be a distinct antibody class until 1966, the true significance of this report has been overlooked. The paper is usually cited only to show that antibodies, but not specifically reaginic antibodies, were made against the tumor. A P-K reaction was also demonstrated by Grace and Dao[73] in a patient with breast cancer and dermatomyositis, and later by Curtis et al.,[49] again in a paitent with dermatomyositis, though this patient had a primary carcinoma in the signoid colon and a metastasis in the lung from which the extract was made. Grace was unable to produce the reaction unless the patient had some sort of cutaneous manifestation of his malignancy.

The existence of a tumor-specific IgE responses is not at all in conflict with the concept that total serum IgE is normal in malignancy. Normal levels of IgE vary widely. For example, the lower limit of normal was one hundred and thirty times less than the upper limit of normal in one study.[72] Obviously a very significant amount of tumor-specific IgE could be produced and total serum levels of IgE might still remain within a normal range.

Only in 1972 was an animal study published proving that a mouse could produce IgE specific for a tumor. Bartholomaeus and Keast[16] achieved this with the B16 melanoma and C57B1/6 mice and also showed that IgE could be produced against alloantigens in a transplant situation.

No data in man or animal has as yet been published to suggest if this IgE response is of benefit to the host.

Some evidence in animals, however, might be related. Parasitic infestations have been shown to act similarly to an adjuvant in stimulating an animal to rpoduce IgE against various antigens.[42] Keller et al.[111] reported that mice infected with Nippostrongylus brasielensis, a nematode, were resistant to growth of a syngeneic mammary adenocarcinoma. In rats, the Walker sarcoma could be inhibted or enhanced depending on the timing of the parasitic infection and tumor challenge. These authors suspected reagin but were unable to demonstrate it. Another study shows that mice with trichinella spiralis have a longer survival and a decreased incidence of spontaneous mammary tumors.[238] Infections with trypanosoma cruzi may similarly affect spontaneous breast cancer in mice. As an adjuvant, Bordetella pertussis augments IgE responses greatly[44] and also has value in tumor immunotherapy in animal[14,137] models and possibly in man.[77] Some speculate that IgE is the factor responsible for prolonged survival.[14,137] For all these observations, IgE, however, has not been proven to be the factor responsible for the tumor resistance and certainly multiple factors have been affected by the experimental manipulations.

The Implications of an IgE Response: Some Hypotheses

At least five distinct but possibly related, hypothetical mechanisms might account for the role of IgE in response to malignancy.

The Gatekeeper Theory

An obvious explanation for the role of IgE is that the release of

histamine and other vasoactive substances triggered by IgE could be causing a vascular change that would hasten tumor expulsion or destruction. As such, IgE or the mediators released from a degranulating basophil might not have a direct effect on the tumor but would act as a catalyst or "gatekeeper",[213] altering vessel permeability and thus, allowing other mediators, immunoglobulins, or chemotactic substances to interact with the neoplasm. Although at first not believed to activate complement, IgE has now been found capable of activating the alternate pathway.[30,100] By virtue of its altering permeability, IgE is said to be responsible for a model of immune complex disease in rabbits.[25] Perhaps the destruction of tissue in immune complex disease is not too dissimilar from the destruction of a malignant clone.

Histamine

A second mechanism for the role of IgE involves histamine. Bourne[37] and Lichtenstein[136] have elegantly reviewed the evidence that histamine, by stimulating cyclic AMP, can modulate immune responses, including basophil degranulation, cell-mediated immunity, antibody production, and neutrophil phagocytosis. In in vitro experiments, histamine retards all of these processes. If IgE can be directed specifically against a tumor, it would be expected that the IgE response would liberate histamine. Indeed, in a rat model it has been shown that after immunization with tumor and B. pertussis adjuvant, tumor challenge seven days later will release histamine from peritoneal exudate cells, presumably via reagin.[220]

If in vivo studies confirm the in vitro work on histamine, the first hypothesis might be that histamine release should potentiate

tumor growth by inhibiting lymphocyte mediated cytolysis. However, histamine seems to have so many possible roles that the inhibition of tumoricidal lymphocytes might be balanced by other positive factors. Histamine receptors have recently been shown to be of at least two types, H-1 and H-2.[31] Conventional antihistamines compete for H-1 receptors such as those responsible for smooth muscle contraction. The receptors for histamine on the basophil and the lymphocyte are of the H-2 type, that is blockable by the experimental pharmacologic agents, burinamide or metiamide. With the characterization of histamine receptors, work should now proceed rapidly elucidating the role of histamine vis à vis the immune response.

Cutaneous Basophil Hypersensitivity

Understanding a third possible interaction between IgE and tumor tissue may come with a greater knowledge of cutaneous basophil hypersensitivity (CBH).[53] This skin reaction to antigen is characterized by a delayed onset in time similar to classic delayed hypersensitivity, but the histology is marked by a high percentage of mast cells or basophils. CBH can be transferred with serum,[11] but since heating the serum to 56°C does not destroy the capacity to transfer,[12] IgE alone would not seem to be the serum factor. Still, with the surface of the basophil marked primarily by IgE, it is hard to believe that the basophil could be involved without the principle immunoglobulin on its surface membrane somehow participating, possibly even in a negative sense.

Further, in animal models CBH has clearly been demonstrated to have a role in tumor rejection.[54] In man the potential for CBH reactions can be monitored by a method called the skin window similar to delayed hyper-

sensitivity skin testing. Antigen is placed over a skin scratch and the area covered by a coverslip. Periodically the cellular exudate collecting on the slip is examined and the percentage of basophils counted. Using this method, Black and Leis[32,33] have found that patients with breast cancer react to their autologous tumor with a basophil-laden exudate. They have found that the percentage of basophils correlates with the sinus histiocytosis in the regional lymph nodes at the time of surgery which in turn correlates with favorable prognosis. The crucial role of the basophil awaits fuller clarification, but surely IgE will be found to have a hand in its work.

The Eosinophil

Fourth, IgE has been closely linked with eosinophilia. Eosinophils are prevalent in allergies and parasitic infections, and a high peripheral eosinophil count is not uncommon with neoplasia.[50,89] The eosinophil produces an enzyme, arylsulfatase, that neutralizes SRS-A[236] and eosinophils may inhibit histamine relase from basophils.[88] Kay and Austen[110] have demonstrated that IgE can cause the release of an eosinophilic chemotactic factor (ECF) and ectopic production of ECF has been shown in a case of carcinoma.[237] In a phenomenon termed radiation-related eosinophilia, patients with pelvic or abdominal carcinoma who receive supervoltage irradiation may develop an eosinophilia which actually correlates with their prognosis.[71] The evidence is only circumstantial but it is tempting to speculate that radiation stimulates IgE perhaps by altering the antigenicity of the tumor or by reducing suppressor T-cells. The increased IgE might cause the eosinophilia and either the IgE itself or the eosinophil might favor the host response over the tumor. Such reasoning is

plausible but highly speculative, and eosinophils, like IgE, may be working toward the patient's detriment rather than his health. Also, although only studied in four patients, total serum IgE has not been found elevated in radiation-related eosinophilia.[243]

T-cell Function

Finally, unraveling the relationship between IgE and the T-cell should be crucial in understanding the role of IgE in response to malignancy. Generally the T-cell is thought to be primarily responsible for cell-mediated immune responses that can aid the host by destroying tumor cells, while a humoral or B-cell response to a tumor can have a variable prognostic effect and may account for enhancement of tumor growth via blocking antibodies.

Besides Hodgkin's disease, other illnesses with decreased T-cell function may have elevated IgE. In infectious mononucleosis, which is frequently characterized by anergy,[143] IgE may be transiently elevated.[168] In sarcoidosis, which is known to be associated with frequent anergy, German investigators found IgE higher than in normal and tuberculous controls.[256] And in atopy, as previously alluded to, while most think of the primary immune dysfunction as increased IgE, several have found decreased T-cell function.

If decreased T-cell function is the primary defect causing elevated IgE, several paradoxes arise. First, besides being responsible for suppressing IgE, the T-cell bears a factor that enhances IgE production,[225] so one must posit that this function is intact while the suppressive function is abrogated. Second, many diseases can be shown to have diminished T-cell function, including commonly most malignancies.[209,246] Why should

not any tumor with depressed cell-mediated immunity have increased IgE? Third, how can measles vaccine simultaneously suppress both atopic symtoms and T-cell function?[265]

Possibly IgE accounts for negative feedback to the T-cell. Acting via histamine or other mediators, it may be IgE capable of suppressing the T-cell as well as the T-cell capable of suppressing IgE.

Conclusions

At present, the relationship between IgE and other functions of the immune system is confusing. No unifying hypothesis has been able to relate IgE, the T-cell, CBH, histamine, and the eosinophil. Consider these puzzles.

Many patients with ataxia-telangiectasia have absent IgE[7,184] and this disease has a high rate of lymphoma at very young ages.[233] On the other hand, some of the highest IgE levels have been recorded for patient's with Wiskott-Aldrich syndrome[29,232] and they, too, develop neoplasia early in life.[233] If IgE abets a host response toward the tumor, why is T-cell function supposedly depressed in atopic eczema and why does histamine turn off tumor cell lysis? Are atopics more likely than others to produce an IgE response toward a tumor? If a tumor-specific IgE response is demonstrable, what effects does it have on the prognosis? What is unique about Hodgkin's disease when other malignancies do not have an elevated IgE?

An intriguing area for studying the role of IgE in response to neoplasia might come from understanding the pathophysiology of dermatologic manifestations of malignancy. Although its role is uncertain, IgE has been implicated in a variety of dermatidides, not just atopic eczema.[47] In diseases like dermatomyositis, erythema multiforme, and pemphigoid,

diseases which may herald an occult malignancy, IgE may prove to be active in the pathogenesis of skin signs. In all the reports of either Grace or Curtis in which P-K reactions were demonstrated for tumor patients, the individuals had some cutaneous manifestation of the tumor. Arbesman has found that IgE is commonly elevated in patients with pemphigoid and in the fluid of its bullae.[8] All of this information suggests that the area of IgE, carcinoma, and skin signs demands further exploration.

While many riddles remain to be solved and questions need to be answered, the role of IgE in response to neoplasia is emerging as a provocative topic for study.

Experimental Design and Rationale

Desiring to clarify the relationship between IgE and neoplasia, we designed experiments to help resolve some of the specific issues raised in this literature review. Some of the studies explored directly the relation between IgE and a tumor; these are described in Part III. Other studies stemmed from a need to understand certain essentials of the IgE response and to perfect a serum assay. These studies were logical preliminaries to those described in Part III and will be considered in Part II. The value of undertaking these preliminary projects can perhaps best be demonstrated by first explaining the projects with a direct relation to tumors.

In Part III, one finds experiments to: 1) demonstrate an IgE response to tumor in animals, 2) evaluate the ability of an animal bearing tumor to make reagin 3) measure IgE levels in children with neoplasms and 4) show that humans can make a tumor-specific IgE response.

Demonstrating IgE responses to tumor in animals was a repetition of the work of Bartholomaeus and Keast, but it was essential in order to evaluate what effect this IgE response had on the course of the malignancy. Evaluating the ability of an animal with tumor to make reagin was designed to answer whether low serum IgE in patients with cancer or high serum IgE in Hodgkin's disease was a cause or effect of the tumor. Measuring IgE levels in children with cancer was a test of the genetic hypothesis that atopics have a selective advantage in resistance to neoplasms. Finally, showing that man can make tumor-specific IgE was a way of duplicating the work of Grace and others without asking healthy volunteers to accept injections of tumor antigen as is done in the P-K reaction.

With these projects in mind, we undertook the preliminary work described in Part II. Obviously in order to do the work on childhood neoplasia, we needed to be able to measure serum IgE. So our work with a RIA for IgE is the first study described. Second, as we were going to be measuring IgE, we wished to be certain that the time of day that the sample was drawn was not influencing the serum level. So ruling out a diurnal rhythm for IgE was a necessary undertaking. Finally we conducted experiments to delineate the ontogenetics of IgE production in the neonatal mouse, that is, the relation of IgE to IgM, G, and A. We realized that such a project would not directly reveal the interplay between lgE and tumor, but understanding the normal physiology of the IgE response will be essential in understanding the factors that determine an IgE response in a patient with cancer. For example, the ontogenetic model that we devised should allow us to study the relationship between IgE and IgA deficiency.

The breast can produce secretory immunoglobulin and evidence suggests that an IgA response may be prognostically significant in women treated with radiation following mastectomy.[161] On the other hand, Grace's work showing P-K reactions in five patients with breast cancer and Black's studies that show that a basophilic response is prognostically significant in breast carcinoma, both suggest that IgE may be important in breast tumors. Our work on ontogeny could help elucidate the interplay of these two secretory immunoglobulins, E and A. Just as the explanation for elevated IgE levels in patients with Hodgkin's disease has derived from studies that furthered the knowledge of the normal physiology of the IgE response but had no direct bearing on tumor research at the time they were performed, so we hoped that our work on the ontogeny of IgE would further our knowledge of the normal physiology of the IgE response and perhaps eventually prove relevant to tumor immunology.

PART II: Basic Studies

(A) Radioimmunoassay

One of the first problems to be solved in order to study IgE was the perfection of a method to assay its concentration in serum. A number of techniques have been described including a solid phase radioimmunoassay (RIA) using sepharose particles (RIST),[105] a RIA using antibody bound to bromacetyl cellulose,[21] a double antibody RIA,[72] radioradial diffusion,[9] immunodiffusion,[102] and mixed immunosorbent RIA.[1] Of the two methods available commercially at the time of this writing, immunodiffusion can only detect elevated levels of IgE. The other, Pharmacia's radioimmunosorbent test (RIST) (Phadebas Test, Pharmacia, Piscataway, N.J.) is a radioimmunoassay essentially identical to that used by Johansson et al. shortly after their having described the first known case of an IgE myeloma.

With practice, the Pharmacia method proved adequate for measuring serum IgE in the high normal to elevated range. However, for values in the range of 10 to 100 units/ml, still within normal limits, counts from standards with known, differing concentrations of IgE began to overlap and values were not reproducible. This is illustrated by the typical standard curve shown in Figure 1. It demonstrates graphically the difficulty in using this assay. Even a group that included Johansson himself has cautioned against the method for measuring low levels of IgE[216] and a comparison study of three methods concluded that the double antibody RIA is the superior method for both reproducibility and assessment of low levels.[185]

FIGURE I

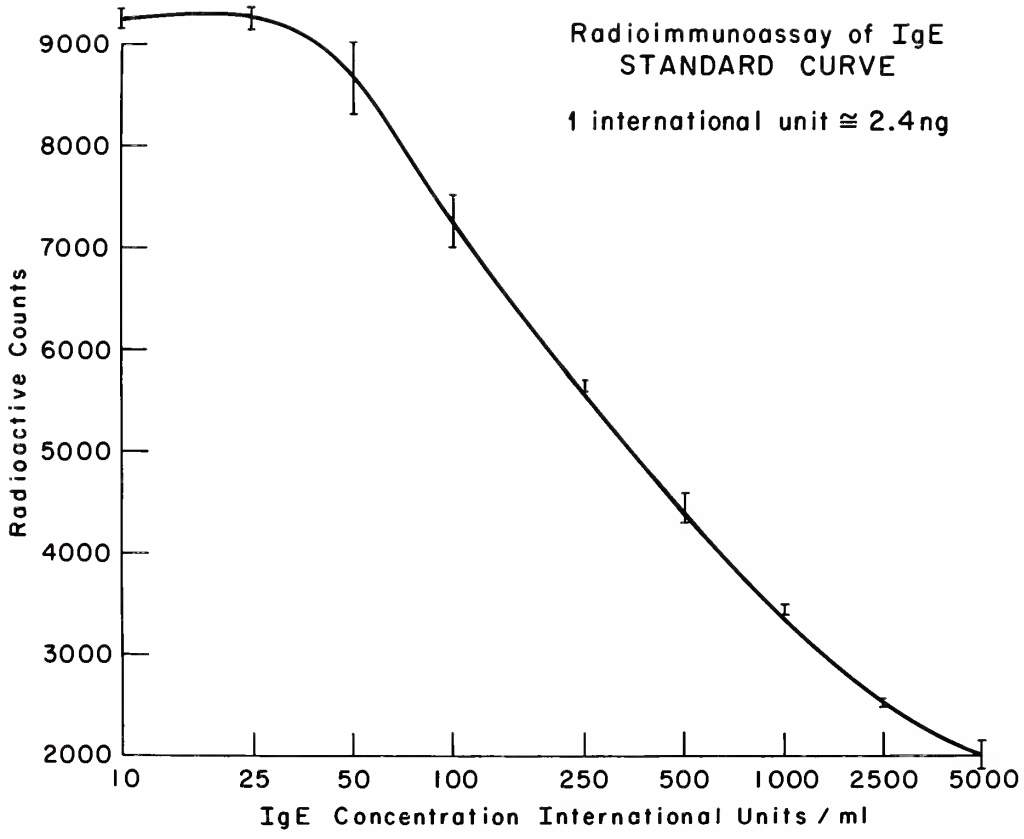
Radioimmunoassay of IgE
STANDARD CURVE
1 international unit ≅ 2.4ng
Radioactive Counts
9000
8000
7000
6000
5000
4000
3000
2000
10
25
50
100
250
500
1000
2500
5000
IgE Concentration International Units / ml

FIGURE I

(B) Circadian Rhythms

Wishing to measure serum IgE, we need to explore the possibility that serum levels might have a diurnal variation. While other immunoglobulins are not known to have daily fluctuations, IgE is unique in being present in such low concentrations, having the shortest half life,[234] and being tissue bound.[101] The symptoms of allergy commonly vary with the time of day. And many factors possibly related to allergic symptomatology do have daily patterns including pollen counts, plasma cortisols, and circulating eosinophil counts.[200] Conceivably, then, serum IgE itself might have a diurnal rhythm, perhaps in atopics if not in normals, and possibly correlating with eosinophil counts.

Multiple determinations, however, were performed on a single unknown sample and counts for an individual determination differed by as many as 1200. With this range of values on a single unknown, it seemed doubtful that the method would be reproducible enough to detect the relatively subtle changes that might occur throughout the course of a day.

Testing the hypothesis of circadian rhythms of IgE must await either 1) demonstration of greater reproducibility of values of unknown sera or 2) perfection of an alternate method to assay IgE.

(C) Ontogeny of the IgE Responses:

The Effect of Anti-Heavy Chain Antisera

Investigating the ontogeny of the IgE response in mice was a major concern of this thesis project. While the results could not directly resolve the relation between IgE and neoplasia, furthering the understanding of IgE production cannot help but bring us closer to elucidating the

role of IgE in host reactions to malignancies.

Kincade and Cooper first described treating chickens with anti-heavy chain immunoglobulin.[113,114] When given *in ovo* injections of anti-mu, that is antisera directed against the heavy chain of chicken IgM, bursectomized chickens not only failed to produce IgM, but also could not synthesize IgG. Working with neonatal, germ-free, Balb/C mice, treatment with anti-mu chain immunoglobulin also prevented production of the immunoglobulin classes,[122,123] M,G, & A. Treatment with anti-gamma chain prevented IgG and IgA responses, but not the response of IgM. Finally, intraperitoneal injections of anti-alpha chain precluded the appearance of IgA, but did not affect IgM or IgG. The explanation offered by the authors is that ontogenetically the mouse's B-cells progress from a sequence of producing IgM to IgG to IgA. The anti-heavy chain antisera interrupts this sequence theoretically either by having a lytic effect on the B lymphocytes or by interfering with their membrane receptors.

This work has been repeated by other investigators in mice that were not kept in a germ-free environment[145,148] and in random bred animals.[166] The effect of anti-heavy chain has also been studied on lymphocytes grown in vitro.[178,179] Studies have confirmed the initial reports with the modification that IgA producing cells may come directly from IgM precursors.[144,151] No reported study has concerned itself with relationships of IgE to the sequential development of immunoglobulins M, G & A, but the question has considerable clinical importance.

Several investigators have measured IgE concentrations in patients with hypogammaglobulinemia, including common variable 'acquired' and Bruton's sex-linked hypogammaglobulinemia.[215,232,184,87,269] Each study has shown that greater than ninety percent of hypogammaglobulinemic sub-

jects have depressed levels of IgE. However, low levels of IgE, as has been mentioned, are difficult to measure. Normal serum levels of IgE are less well established and vary more widely than the normal levels of other immunoglobulins. Subjects who are apparently completely normal and who have absent IgE have been reported.[132,184] Possibly some of these depressed levels of IgE in these hypogammaglobulinemic subjects are within normal range.

Several pieces of evidence suggest that IgE might have an ontogenetic development independent from the M to G to A progression. First, Ishizaka et al. have studied the production of IgE in vitro in primed lymphocytes treated with anti-mu sera.[95] These lymphocytes were able to produce IgE despite the presence of anti-heavy chain sera and the reduction of other immunoglobulins. However, because this work was done with primed lymphocytes, i.e. lymphocytes that had been exposed to antigen prior to the treatment with anti-mu and re-exposure to antigen, the study merely indicates that the cells producing IgE were unaffected by anti-mu but says nothing about the possible ontogenetic precursors of these IgE-bearing cells.

Second, although acquired hypogammaglobulinemia is not a single entity, but a group of disorders with a variety of causes,[2,69] some hypogammaglobulinemic subjects retain IgE despite loss of other immunoglobulin classes. In an experimental model in rabbits, it is possible to stimulate IgE without detectable antibodies of other classes appearing.[268] In doing immunofluorescence studies on lymphoid tissue, Tada and Ishizaka reported on one hypogammaglobulinemic subject who appeared to retain a normal number of IgE-bearing lymphocytes despite markedly depressed

plasma cells or B-cells of other immunoglobulin classes.[221] Two other similar patients were devoid of IgE as well as IgG, M, or A bearing cells. A recent case report describes a hypogammaglobulinemic patient who retained normal levels of IgE and suffered from a malabsorption syndrome.[128]

Third, many patients with hypogammaglobulinemia develop anaphylactoid symptoms when being treated with passively administered gamma globulin.[59] Both these observations suggest that the synthesis of IgE may be preserved while other immunoglobulins are absent. Schumacher and Ellis,[201] however, have studied several hypogammaglobulinemics and demonstrated that their basophils do not release histamine in the presence of foreign gamma globulin indicating that the anaphylactoid responses may not be due to IgE-mediated reactions.

Fourth, Gajl-Peczalska et al.[67] reported a case of Bruton's sex-linked agammaglobulinemia in which the subject had demonstrated allergic symptoms and had apparently a normal number of circulating IgE-bearing lymphocytes while B-cells of other immunoglobulin classes were virtually absent. Because of the possible confusion between basophils and IgE-bearing lymphocytes when studied by immunofluorescence, the conclusions of this study have been questioned.[195] A second case report[78] describes another child with Bruton's hypogammaglobulinemia who had positive immediate hypersensitivity skin tests and who reacted with a wheal and flare to injections of anti-IgE, thus demonstrating the presence of IgE on his mast cells despite absent serum levels of other immunoglobulins.

Finally, occasionally patients with severe combined immunodeficiency, a condition with depressed T-cell as well as B-cell function, will have

an elevated serum IgE,[267] while other immunoglobulin levels are typically depressed.[85]

With this conflicting evidence relating to the ontogeny of IgE, we studied the production of IgE in mice treated with anti-mu sera from birth.

Methods:

Antisera- Goat anti-mouse mu sera (lot 40667 and/er lot 40900) was purchased commercially from Meloy laboratories, Springfield, Virginia. An absence of anti-light chain activity, kappa or lambda, or anti-heavy chain activity other than anti-mu was demonstrated with Ouchterlony gel diffusion plates. The purchased antisera was preserved with sodium azide and was dialyzed against sterile saline to remove this preservative prior to injection into animals. Control animals were injected with bevine gamma globulin (BGG), Cohn Fraction II, Sigma Chemical Co. BGG was dissolved in sterile saline at a concentration of 10 mg/ml, passed through a 0.20 micron millipore filter, and stored in sterile glass syringes until ready for use.

Animals- Mice were provided from the specific pathogen free colony of the Biobehavioral Science Research Center of the University of Connecticut, Storrs, Connecticut, at a nominal cost. In the preliminary work, DBA/1 mice were selected for treatment with anti-mu as these mice are especially reactive to evalbumin (OA), an antigen for which IgE antibodies can be readily demonstrated by passive cutaneous anaphylaxis (PCA) (see below).

A number of difficulties forced us to abandon the use of DBA/1 mice. First as mothers, these mice are particularly temperamental and it was necessary to use surrogate C57Bl/10 or C57Bl/6 lactating females.

Second, the animals produce small litters. Third, the animals are relatively small in size. And fourth, these mice were especially sysceptible to stress and infection. Although we began with roughly forty DBA/1 infants, only seven (four experimentals and three controls) were able to survive to the end of the experiment thirty-four days after birth.

Of these experimental animals that did survive, all were runted when compared to the controls. The weights of the two groups were statistically different by Students' T test, $p < 0.0001$. Thus, it was impossible to tell if the immunoglobulin titers produced by the experimentals reflected the effect of immunosuppression by anti-mu or the effect of the runting itself.

These problems were minimized in subsequent experiments with the use of the first generation products of the mating of DBA/1 males and C57Bl/10 females. These animals had a number of advantages. The litter size was larger. The animals were healthier. The need for surregate mothers was obviated. And the reactivity of these animals to OA was at least as great as the reactivity of the DBA/1s.[230]

Male and female six week old Swiss mice from the Sterling Hall of Medicine colony, Yale University Medical School, were used as recipients of sera to titer IgG1 antibodies by PCA. Preliminary work had shown these mice to be comparable to CFW mice (Carworth Farms, New City, New York). CFW are a standard breed used by other investigators for PCA work.[131] Two hundred gram male Sprague-Dawley rats were purchased from Charles River Laboratories, and used as recipients for IgE titration by PCA as described by Mota and Wong[164] and Konig et al.[119]

Injection schedule- The schedule for treatment with anti-mu varied slightly from litter to litter, but in most cases, neonatal DBA/1 x C57Bl/10 mice received their first injection, either antisera or BGG, within twenty-four hours of birth. This was considered to be day one. Animals were given 0.05 cc on days one through four intraperitoneally (i.p.) and 0.10 cc on days 9, 16, and 26. The total dose was therefore 0.50 cc. On day 21 all animals were given an i.p. injection of ovalbumin (OA) (twice recrystallized, Worthington Biochemical, Freehold, N.J.) 0.1 ug dissolved in saline plus alum, 1.12mg, in a total volume of 0.1cc. The alum was prepared as described by Levine and Vaz[131] and had a dry weight of 28 mg/ml. On day 34, thirteen days after exposure to antigen, all animals were placed under light ether anesthesia and bled repeatedly through the retro-orbital plexus with glass pipettes. Approximately 0.20 cc of serum was obtained per animal.

Titration of immunoglobulin levels-

A) PCA: This technique has been well described by Ovary[177] and others.[44] [131] In brief, mouse sera contains two immunoglobulin classes of reaginic antibody, IgE and IgG1.[34,189] Reaginic antibodies can be thought of as those immunoglobulins that can passively sensitize the surface of mast cells or basophils and which cause the release of serotonin, histamine, and/or other vasoactive substances from these cells when exposed to appropriate antigentic stimulus. Unlike IgG1, IgE activity can be destroyed by heating at 56°C or by treatment with 2-mercaptoethanol.[34] In mice, activity of passively injected IgE will persist after 48 hours by which time essentially all IgG1 activity has disappeared.

To titer IgG1, sera was diluted with saline in a two fold manner

beginning at a concentration of one to five. The backs of Swiss mice were shaved and after light ether anesthesia, 0.03 cc of diluted serum was injected intradermally into the shaved surfaces. Up to four injections of varying dilutions were given per back. After two hours, the recipient mouse was again given ether anesthesia and 0.15cc of saline with 0.7% CA and 0.4% Evans blue (Matheson, Coleman, and Bell, E. Rutherford, N. J.) was injected intravenously via the retro-orbital plexus. One half hour after that, the animals were sacrificed and reactions read by removing the skin from the back of the animal and observing the blueing at the injection site. Theoretically immunoglobulin from the passively administered serum will sensitize the mast cells in the recipient animal. When appropriate antigen is given, the mast cell will degranulate, releasing histamine and/or serotonin. This alters vascular permeability and allows the extrusion of Evans blue, causing the local blueing which could be easily read as a positive reaction. The titer of antibodies was considered to be the highest dilution of serum causing blueing of 5 mm or more in diameter in at least two of three mice. If a dilution of 1:5 failed to produce a reaction in a majority of mice, the titer was considered to be zero.

Titration of IgE antibodies in mouse sera can be done in either of two ways by using PCA. Recipient animals can either be mice or rats. For the mice, administering antigen i.v. 48 to 72 hours after the skin has been sensitized will detect essentially only reagin of the IgE class. An alternative method is to use rats, as mouse IgE, but apparently not IgG1, will bind readily to rat mast cells.[119]

Male Sprague-Dawley rats were anesthetized with sodium pentobarbital (Diabutal, Diamond Labs, Des Moines, Iowa) approximately 0.80 cc/kg i.p. The back of the rat was shaved and up to 24 injections of 0.05 cc of varying dilutions of sera were given per animal. Two hours later tha animal was anesthetized with ether and injected i.v. through the dorsal vein of the penis with 1 cc of saline containing 0.4% Evans blue and 0.7% OA. Half an hour later animals were sacrificed under ether and their dorsal skin removed. The titer was considered to be the highest dilution causing blueing of at least 5 mm in two of three animals.

B) Ouchterlony plates: Presence of IgG1 and IgG2 was determined by appearance or absence of a precipitation line when serum diluted 1:5 was allowed to raact on a gel diffusion plate with the appropriate class specific antiserum provided by Dr. Philip Askenase. This method is described by Arnason.[10]

C) Passive hemagglutination: IgM and IgG antibody levels to OA were measured by their ability to agglutinate sheep red blood cells (SRBC) coated with antigen. Blood was defibrinated and the cells washed. Red cells were removed and mixed for four minutes at room temperature with 3 mg 0.1% $CrCl_3.6H_2O$ (Malinckrodt Chemical, St. Louis) and 3 ml of OA at a concentration of 10 mg/ml. The treated cells were washed five times and resuspended in normal saline at a concentration of 5%. This suspension was diluted 1:5 in phosphate buffered saline.

Serum to be evaluated for antibody was tested in serial two fold dilutions beginning at a dilution of 1:10. Rabbit hyperimmune antisera to OA was provided by Dr. Philip Askenase and used for control. Untreated

serum was considered to reflect both IgM and IgG antibodies. To measure just IgG, serum was reacted with mercaptoethanol (Pierce Chemical Company, Rockford, Ill,) and tested at dilutions beginning with 1:20. To 0.1 ml of serum to be tested, 0.3 ml of 0.133 M mercaptoethanol was added and incubation carried out at room temperature for two hours.

Serial dilutions of serum were added to autotiter hemagglutinating plates containing the treated SRBCs and the agglutination end point read. Antibody titers are expressed as the dilution for which agglutination ended. Fifteen dilutions were tested for each sample. If no agglutination occurred at the lowest dilution tested, the titer was considered to be zero.

Splenic histology- Spleens were removed immediately after sacrifice and frozen in liquid nitrogen. Spleens were stored at -90°C until ready for sectioning and staining. Microtome sections of four to six microns were prepared. Tissue was stained with hematoxylin and eosin and evaluated with light microscopy.

Animal survival precautions- Obviously the survival of the animals was crucial in the performance of this experiment. Absorption of injections without rupture of bowel is a delicate technique in a newborn. These injections were skillfully given by Sandra Rosenbaum. To minimize the risk of cannabalization, we handled all animals with gloves, and during the first few days of life, rubbed the newborns' backs with Vicks Vapo Rub (Vicks Chemical Co, New York City, NY) immediately after injection in an effort to discourage the mother from injuring the litter and to minimize odor from handling.

Results:

The results of treating DBA/1 X C57BL/10 mice with anti-mu antisera and then immunizing with OA are given in Table II. Mean values and standard deviations for the BGG treated and the anti-mu treated groups are given in Table III.

Immunosuppression clearly was achieved. By Students' T test, the difference between the two groups for IgG1 values as determined by PCA was significant with $p < 0.025$. Similarly, comparing the IgM and IgG values as determined by PHA showed $p < 0.005$. $P < 0.01$ for the comparison of IgG levels as measured by PHA. In contrast, the IgE values did not differ significantly between the two groups ($p > 0.3$).

Splenic histology is pending.

TABLE II. RESULTS OF ANTI-MU TREATMENT ON IMMUNOGLOBULIN PRODUCTION

Litter	Animal	Treatment	Weight (gms.)	Sex	IgE by PCA	IgG+IgM by. PHA	IgG by PHA	IgG1 by PCA	IgG1 by Ouchterlony[1]	IgG2 by Ouchterlony[1]
1	A	ANTI-MU	5.8		0	0	0	0	+	0
	B	ANTI-MU	9.3		40	0	0	0	+	0
	C+D[4]	BGG	4.2 5.2		20	0	0	0	N.T.[5]	N.T.[5]
	E	BGG	10.8		1280[2]	5000[3]	640	160	++	++
2	A	ANTI-MU	15.0		160	80	0	0	±	±
	B	ANTI-MU	19.0		40	0	0	0	+	±
	C	BGG	18.2		320	1280	80	5	+	±
	C	BGG	16.9		160	640	40	0	+	±
3	A	ANTI-MU	14.9		320	320	80	0	±	±
	B	BGG	13.6		640	5000[3]	1280	80	+	±
	C	ANTI-MU	14.2		1280[2]	160	0	10	0	0
	D	ANTI-MU	16.3		320	10	0	0	0	0
	E	BGG	15.7		80	10,000[3]	1280	40	+	0
	F	BGG	14.9		80	5000[3]	320	40	+	0
4	A	ANTI-MU	17.0		640	1280	160	40	+	0
	B	BGG	15.8		1280[2]	5000[3]	160	160	+	+
	C	ANTI-MU	15.9		640	160	0	0	+	±

1 All sera tested at 1:5 Dilution
2 Not tested at dilution greater than 1280
3. Values over 5000 are rounded to the nearest thousand
4 The small size of these two animals necessitated combining their sera for titration
5 NT = Not tested

TABLE III MEAN VALUES AND STANDARD DEVIATIONS FOR IMMUNOGLODULIN LEVELS OF ANTI-MU AND BGG TREATED MICE

Treatment	IgE by PCA	IgG1 by PCA	IgG + IgM BY PHA	IgG by PHA
Anti-Mu	382 ± 391	5.6 ± 12.6	223 ± 387	27 ± 53
BGG	482 ± 495	60.6 ± 62.7	3990 ± 3055	475 ± 502

Discussion:

The data indicate that while treatment with anti-mu produced a statistically significant reduction in anti-OA antibodies of the IgM and IgG class, anti-OA of the IgE class was not similarly affected. The lack of dramatic differences between the two groups in total serum IgG1 and IgG2 as measured by Ouchterlony technique can be explained by maternal antibodies, especially as animals were either not weaned or weaned just prior to the termination of the experiment.[145]

While a number of clinical and experimental observations on individuals with hypogammaglobulinemia as referred to earlier might have suggested this result, the outcome nonetheless seems counter to the fact that most hypogammaglobulinemic persons have a significant reduction in IgE.

Another piece of experimental evidence would certainly seem to suggest that anti-mu treatment would decrease serum IgE. Taniguchi and Tada have recently shown that a solubilized fraction of T-cells can serve a helper function in immunoglobulin production.[225] This helper molecule can be absorbed by anti-mu. They believe that the helper activity may be an IgT, an immunoglobulin on the surface of the T-cell. Unlike IgG, passively administered IgM in small amounts acts to enhance the production of immunoglobulin[83] including IgE.[218] One speculation would be that this effect is mediated by priming helper T-cells.

One would expect then that treatment with anti-mu would exert a

significant effect on IgE production if only by virtue of its potential action on T-cells.

Abundant evidence, however, indicates that the effect of anti-mu is independent of T-cells. In in vitro spleen cell cultures, T-cells can be removed, but the effect of anti-mu will not be diminished.[178] Anti-mu antibodies have a suppressive effect even if the antigen is thymus-independent such as ferritin.[123] Anti-mu antibodies can prevent the transplantation of such B-cell tumors as Friend leukemia virus leukemia[149] or myelomas.[146] And anti-mu does not effect such T-cell functions as skin graft rejection.[147] and possibly helper function itself.[265]

Still specific T cell functions may be attributable to subpopulations of T-cells[205,257] and a subset responsible for assisting or suppressing the ontogenetic development of the B-cell line might be affected by anti-mu while other T-cell functions are undisturbed. If work substantiates that some T-cells bear an IgT with mu chain determinants,[225,261] one would predict that anti-mu treatment should affect T-cell function, including helper function in the production of IgE. In our work, either helper function was unaltered or a second effect of anti-mu compensated for diminution in helper function and permitted normal serum IgE levels.

While treatment with anti-mu suppresses IgM, IgG, and IgA, a return to normal levels of IgA despite prolonged treatment with antisera has been reported.[145,148] It is unlikely that such a rebound is also occuring with IgE because the return of IgA values was observed only

after six weeks of treatment whereas at three weeks, the crucial time in this experiment when animals were exposed to antigen, IgA levels were always still markedly depressed. Since, however, the timing and extent of the reappearance of IgA is dependent on the dose of anti-mu,[146] we are conducting studies to determine if increasing the dosage of antiserum might result in a significant depression of IgE.

This experimental model utilizing anti-heavy chain immunoglobulin has potential for elucidating other aspects about the physiology of IgE. For example, the question of affecting IgE levels by selectively suppressing IgA is especially relevant as some have suggested that the true immunological defect in atopy is not an excess of IgE but a transient deficiency of IgA, which may be the only truly significant blocking antibody.[217,226] As IgE and IgA may both be important factors in the immune response in breast carcinoma (see p. 19), this work could help elucidate the relationship of these two immunoglobulins in response to malignancy.

Treating mice with anti-gamma 1 and/or anti-gamma 2 may help clarify the relation between IgG and IgE. A tradiational view holds that IgG is the principle blocking antibody and that IgG levels may be inversely related to IgE. Support for this hypothesis came when Tada and Okumura showed that rats given IgG passively produced markedly depressed IgE titers.[222] However, this report could not be reproduced by the Ishizaka and Okudaira[96] in mice, nor could the Ishizakas show an inverse relationship between IgG and IgE levels during the course of long term hyposensitization immunotherapy.[91] By treating slightly older

mice with anti-gamma we might be able to inhibit IgG selectively and analyze the relationship between 'blocking' IgG and IgE.

Thus, results so far indicate that synthesis of IgE is either independent of the ontogenetic progression of other B lymphocytes from IgM to IgG to IgA or IgE may actually precede IgM in ontogeny. Current work is in progress to verify these results, especially with higher doses of immunosuppression, and to elucidate the effect of anti-alpha antiserum on IgE production.

PART III: Tumor-Related Studies

(A) IgE Responses to Tumor in Animals

As discussed in the literature review, Bartholomaeus and Keast in 1972 reported that mice could make IgE responses to tumors.[16] We attempted to repeat their work as a preliminary to answering some of the following questions: Does an IgE response alter the course of the malignancy? Could passive transfer of IgE affect tumor challenge or the course of an already transplanted tumor? Is the immunotherapeutic effect of certain adjuvants like B. pertussis related to is effect on IgE response? While Bartholomaeus and Keast had shown that IgE could be produced, they had not addressed themselves to any of the above questions.

To assess the role of IgE we attempted to produce a variable antibody response by using a range of immunizing doses of tumor and two different adjuvants.

Methods:

Animals- Male and female AKD2F1/J, DBA/2J, and C57Bl/6J mice were purchased from Jackson laboratories, Bar Harbor, Maine. Swiss mice were obtained from the Sterling Hall of Medicine colony, Yale Medical School. CFW mice were purchased from Carworth Farms, New [illegible] City, New York. Lewis rats were purchased from Microbiological Associates. Recipient animals for PCA titrations were either Swiss or CFW mice

or the rats. The Lewis rats had been shown to be comparable to Sprague-Dawley rats in PCA reactions to OA as described in the previous section.

Tumors- A murine lymphoma, L5178Y, maintained in vivo was provided for us by Dr. Robert Capizzi. This tumor was initially reported in DBA/2 mice and in our hands took readily in AKD2F1/J mice at i.p. doses of 10^3 to 10^6 cells. Death preceded by ascites occurred regularly in 10 to 18 days depending upon the number of malignant cells initially injected. This tumor was selected because some immunological aspects of its growth had previously been studied[6,60,61,244] and it was clear that injecting animals with irradiated cells could produce immunity.[6]

The murine melanoma, B16, was given to us by Dr. Ted Reid, who maintained an in vitro cell line. This tumor took readily in C57Bl/6 mice and caused death in 17 to 35 days. It is the same tumor as initially reported upon by Bartholomaeus and Keast.

Immunization of animals and serum collection- For the work with L5178Y, a variety of mice strains including AKD2F1/J, DBA.2J, CFW, Swiss, and C57Bl/6J were given injections of tumor in doses ranging from 1.4×10^1 to 2.8×10^6 cells. The cells were usually viable, but in some instances they were irradiated prior to injection. Either an i.p. or a subcutaneous (s.c.) route of injection was used. Cells were either given without adjuvant, with 1 mg of alum, or with killed B. pertussis organisms (Eli Lilly and Co., Indianapolis, Ind.) with the number of bacteria varying from 9.6×10^6 to 4.4×10^9 depending on the trial. Alum was prepared as previously described.[131] Mice were bled from seven to nineteen days after an

an initial injection of cells or following a booster injection of tumor.

For work with the B16 melanoma, 6 C57Bl/6J mice given a s.c. injection of 3×10^5 cells plus 1 mg. of alum. Serum was obtained from these mice one and two weeks after immunization.

For all animals, bleedings and i.v. injections were usually performed with the animal under light ether anesthesia.

Irradiation- When cells were irradiated, a Semans x-ray machine was used with a 2 Al filter. When set at 250 KV and 15 mA, this machine delivers 500 rads/min. Three to five thousand rads were given cells depending on the trial.

Passive Cutaneous Anaphylaxis (PCA)- PCA reactions were performed as previously described with recipient animals including Swiss and CFW mice and Lewis rats. Antigen prepared from tumor cells was given in the solubilized forms as described below. Antigen was usually given i.v., but in some trials the antigen was given intradermally (i.d.) directly into the site that had previously received serum passively. When antigen was given in this manner, a control injection of antigen into unsensitized skin was also tested.

Active Cutaneous Anaphylaxis (ACA)- In a few instances, antibody response was assayed by injecting an animal with antigen i.d. some time after that animal had been actively immunized with tumor cells. The animal receiving the i.d. injection would first be shaved, receive the injection of intradermal antigen, then immediately receive an injection of Evans blue with saline i.v. Thirty minutes later tha animal would be sacrificed and reactions judged from the underside of the skin.

Control reactions included injection of saline instead of tumor antigen and injection of tumor antigen into non-immunized animals. Positive reactions may be attributable to either IgE or IgG1.

Solubilization of tumor antigen- A positive reaction in PCA requires that antigen reaction with IgE on the surface of a basophil or mast cell. For steric reasons, we believed that this reaction would be difficult to elicit without first solubilizing the tumor antigen. Although in a few instances whole tumor cells were used to attempt to elicit the PCA reaction, we generally solubilized the antigen by one or more of the following procedures: rapid freezing and thawing in a dry ice-acetone bath; ultrasonication with a sonifier cell disruptor, Model W185D, Heat systems-ultrasonics, Inc.; ultracentrifugation at 30,000 RPM or at 30,000 g for one hour; irradiation with three to five thousand rads; and extraction with 3 M KCl as described by Meltzer et al.[162] In order to elicit PCA reactions, antigen was administered i.v. via the retro-orbital plexus in the mouse or via the dorsal vein of the penis in the rat. Either Evans blue was added directly to the tumor antigen or a second injection of saline plus Evans blue was given immediately before or after the injection of tumor antigen. An initial concentration of greater than 10^6 cells/ml. and usually greater than 10^7 cells/ml was always used to begin the solubilization process.

Foot pad testing- An index of delayed hypersensitivity response to L5178Y was assessed by injecting appropriate antigen into the foot pad of immunized mice. Antigen injected into one foot was 0.04 ml of irradiated cells at a concentration of 1.25×10^7 cells/ml. The other foot pad received 0.03 ml of tumor antigen extracted with 3 M KCl. Foot pad thick-

ness was measured prior to injection and twenty-four hours after antigen. Mice tested included 3 AKD that had received 10^3 L5178Y plus 10^8 B. pertussis, 3 Swiss that had received 10^3 L5178Y twice with two weeks separating the immunizations, and 3 Swiss mice that had received no exposure to tumor. Mice that had been immunized were tested twenty days after the initial injection of tumor.

Results:

Using the tumor, L5178Y, despite the variety of animals immunized, the range of bleeding times, the types of adjuvants, the varied routes and doses of immunogen, and the alternative methods for solubilizing the antigen, we were unable to produce consistent PCA reactions for either IgE or IgG1.

Results are summarized in Tables IV and V.

A description of a typical experiment should clarify the procedure followed.

Five groups of four AKD2F1/J mice were given i.p. injections of irradiated (3000 rads) L5178Y cells in doses ranging from 3×10^1 to 3×10^5 cells. Cells were given with 1 mg of alum in an i.p. injection of 0.2 cc. Although 3,000 rads has been reported to destroy the activity of L5178Y,[6] by day 18, the majority of animals having received the maximum tumor dose were dead. The survivors of this group were bled on this day and all other groups were bled the next day. Mice in all other groups showed no signs of illness and were disease free for at least an additional six weeks. Serum was pooled and frozen at -20°C until ready for use. L5178Y cells in a concentration of 9.6×10^6 cells/ml. were

TABLE IV PCA REACTIONS TO L5178Y FOLLOWING PRIMARY EXPOSURE TO TUMOR ANTIGEN

Mouse Strain and Number of Animals	Tumor immunizing Dose (cell#) ?adjuvant ?irradiation	⇐Day after immunization serum collected		PCA test antigen(s)	Comments
4AKD	3 x 10^1 l.p. +alum irradiated	19	1	Freeze/thaw x 3; Ultrasonicate; Utracentrifuge 30,000 RPM	No morbidity from tumor
4AKD	3 x 10^2 l.p. +alum	19			No morbidity from tumor
4AKD	3 x 10^3 l.p. +alum	19			No morbidity from tumor
4AKD	3 x 10^4 l.p. +alum	19			One of three animals ⊕ for IgE at 1:3 Dilution No morbidity
4AKD	3 x 10^5 l.p. +alum	19			No morbidity from tumor
5AKD	2.5 x 10^2 l.p. + Pertussis irradiated	18			Animals dead or moribund by Day 18
3AKD	10^3 l.p.	7	1	Whole cells, 10^8/ml	Animals dead Day 15
3AKD	10^3 l.p. + 10^8 pertussis	7	2	Irradiated cells	No morbidity from tumor
			3	Sonicated cells	
3 SWISS	10^3 l.p.	7			No morbidity from tumor
3 SWISS	10^3 l.p. + 10^8 pertussis	7			No morbidity from tumor
3AKD	2.8 x 10^6 l.p.	9	1	Freeze/thaw x 3; Ultrasonicate; Ultracentrifuge 30,000 g	Also negative at 14 days by ACA
4AFW	1.4 x 10^3 l.p.	9			Also negative at 14 days by ACA
4CFW	1.4 x 10^2 l.p.	9	2	Whole cells	Also negative at 14 days by ACA
4CFW	1.4 x 10^1 l.p.	9			Also negative at 14 days by ACA
5C57Bl6	10^6 + alum S.C.	10	1	Freeze/thaw x 5	Minimum dilution tested 1:2; No morbidity from tumor
4DBA/2	10^6 + alum S.C.	8to10			Minimum dilution tested 1:2; Death 12 days
6CFW	10^6 + alum l.p.	10			Minimum dilu. tested 1:2; No morbidity from tumor
3AKD	10^3 + 10^8 pertussis l.p.	14	1	Irradiated cells	No morbidity from tumor
			2	KCl extract	
3AKD	10^3 l.p		3	Intradermal KCl Extract	Animals dead Day 15

TABLE V PCA AND ACA REACTIONS TO L5178Y FOLLOWING SECOND EXPOSURE TO TUMOR ANTIGEN

Mouse Strain and Number of Animals	Initial immunization Cell #	Day of Boost	Booster Dose	Day after Boost Serum Collected	PCA Test Antigen	ACA Test Antigen
4AKD	3×10^1 +alum irradiated l.p.	28	Repeat Initial Dose	19	1 Freeze/thaw x 3; Ultrasonicate; Utrocentrifuge @ 30,000g for 1 Hour	
4AKD	3×10^2 +alum irradiated l.p.	28	Repeat Initial Dose	19	2 Whole cells	
4AKD	3×10^3 +alum irradiated l.p.	28	Repeat Initial Dose	19		
4AKD	3×10^4 +alum irradiated l.p.	28	Repeat Initial Dose	19		
4AKD	2.5×10^2 irradiated $+1.9 \times 10^7$ pertussis l.p.	28	2.25×10^2 4.4×10^9 pertussis l.p.	19		
3AKD	$10^3 + 10^8$ pertussis l.p.	24	2.8×10^6 l.p.	9		
3 SWISS	10^3 l.p.	12	Repeat Initial Dose			1 Irradiated Cells 2 KCl Extract Both 12 Days Following Boost
3 SWISS	$10^3 + 10^8$ pertussis	12 12	Repeat Initial Dose			

solubilized by freezing and thawing three times, ultrasonicating with the sonifier intensity set at 4.5 and 7 bursts of 3 to 5 seconds given to the suspension of cells surrounded by an ice water bath, and ultracentrifuging at 30,000 RPM for one hour. The supernatant was used as the antigen to elicit PCA reactions.

Each of the Swiss and CFW mice recipients for PCA titration received 0.1 ml. of antigen i.v. This administration was given either two or 48 hours after the animal had been passively skin sensitized with a dilution of serum of at least 1:5 from the actively immunized animals. With one exception no positive reactions were elicited. The one exception occurred after a 48 hour sensitization period at a dilution of 1:5 with serum from the group that had received 3×10^4 cells, but as this reaction occurred in only one of three animals, it was not scored as positive when judged by criteria generally used for scoring PCA reactions, namely a positive response in the majority of animals tested.

ACA responses in Swiss mice that had received 10^3 leukemic cells 26 and again 12 days previously were tried with irradiated cells and with the hypertonic KCl extract. Reactions in the immunized mice did not differ from controls.

Experimentation with the B16 melanoma also did not produce consistently psoitive results. As described in the methods section, 6 C57 mice were immunized with a s.c. dose of melanoma and alum and then bled 7 and 14 days after immunization. The sera was pooled from each bleeding and diluted 1:5 with saline. Tumor antigen was solubilized by harvesting

5×10^6 cells growing in vitro and freezing and thawing them rapidly five times in a dry ice-acetone bath. Four Swiss mice were skin sensitized and two hours later each received an injection of Evans blue i.v. followed by 0.15 cc of the solubilized antigen. In each case the mouse receiving antigen died within ten minutes of the intravenous injection of an apparent cardiorespiratory arrest. Death was clearly not due to the ether anesthesia that had been used to facilitate the giving of i.v. injections as usually the animal was visibly recovering from the ether before respiratory difficulties developed. Death was also not due to the volume of fluid administered as mice in our hands had been shown to tolerate far greater fluid loads quite well. Pulmonary emboli from particles in the antigenic suspension was considered a possibility, but centrifuging the antigenic fluid to remove possible particles did not seem to affect the toxicity of the challenge antigen. This antigenic suspension was also lethal to animals that had received no passive serum.

Thirty-four days after receiving the s.c. melanoma injection, three of the survivors received an i.d. injection of tumor cells that had been ultrasonicated. One of the three animals produced a reaction markedly larger than the response produced by a similar injection into unimmunized controls.

Discussion:

While the lack of consistent positive results prevented us from assessing the effect of an IgE response on tumor growth in an animal system, useful information did emerge from this work.

In their report on mice producing an IgE response to the B16 melanoma, Bartholomaeus and Keast solubilized the tumor antigen by a process of freezing and thawing, ultrasonication, and ultracentrifugation.[16] While we wished to duplicate their work as closely as possible, we were loathe to ultrasonicate and ultracentrifuge the tumor cells. Ultrasonication, if done at too high a frequency, can serve to destroy antigenicity rather than solubilize it.[108] Unfortunately, in their report Batholomaeus and Keast did not specify the frequency with which they sonicated. We also reasoned that ultracentrifugation would only cause the loss of some antigen. For these reasons, in most of our work we solubilized the B16 cells only by rapid freezing and thawing, and the intravenous administration of this suspension proved lethal to passively sensitized animals.

A personal communication from Bartholomaeus clarified some of our difficulties.[17] First, the frequency of their ultrasonication was reported as ranging from 16 to 24 KCS. As the ultrasonifier that we employed performed at 20 KCS, ultrasonication would probably not have destroyed antigenicity. The positive active cutaneous anaphylaxis response obtained in one of three animals using ultrasonicated antigen is encouraging and confirms the idea that sonication would not destroy all antigenicity.

Second, the author reported that while they had been successful in demonstrating IgE responses with a variety of tumor systems, they had been unsuccessful with all leukemias and lymphomas. Hence, our failure with L5178Y despite careful adherence to the methods of Bartholomaeus and Keast in some of the trials with this tumor becomes understandable.

In trying to interpret our negative results, we could not be sure if they arose from a failure of the immunized mice to produce an antibody response or from a failure of our antigen to be solubilized. The communication from Bartholomaeus re-emphasized the necessity of using some solubilization procedure to elicit the PCA. We had some evidence that mice were making an immune response to the tumor, namely the mouse's ability to survive tumor challenge. For example, 3 AKD2F1/J mice were given 10^3 L5178Y i.p. and three were given an equal number of tumor cells plus 10^8 heat killed Bordetella pertussis. 16 to 18 days after this injection, the animals from the first group were dead. Eight weeks after the injection the animals that had received tumor plus B. pertussis showed no evidence of disease. As shown in Table IV, despite the acquired immunity, we were unable to demonstrate the presence of either IgG1 or IgE antibodies directed against the tumor in these mice. Foot pad testing likewise failed to demonstrate the acquired immunity.

While B pertussis has a variety of effects on the immune system, many investigators have utilized its potent adjuvant effect on IgE responses. Furthermore, several authors have noted that like bacille

calmette guerin[19,76,127] and corynebacterium parvum,[48] B. pertussis may be used to prolong survival in animals bearing tumors.[13, 14,137,247] It has also been tried in immunotherapy of human neoplasia.[77] Like other immunotherapeutic agents,[180] timing of its administration in certain ways may also enhance tumor growth.[249] Some authors have speculated that the effect of pertussis on IgE and the effect on survival after tumor challenge might be related. With this in mind, to attempt to demonstrate IgE in pertussis immunized animals seemed logical. Negative results, especially as all the work with pertussis was done only in the lymphoma system, do not rule out the possibility that IgE might be an important factor in immunotherapy with B. pertussis.

An outgrowth of these experiments was the observation that when the B16 melanoma was admixed with alum prior to its inoculation, the survival time of the mice seemed to be prolonged 50%. The alum was probably not toxic to the tumor cells prior to the injection, as the alum was buffered and in an isosmotic medium, although this possibility was not ruled out by examining the viability of tumor cells combined with alum in vitro. Alum itself might prove to have some use in tumor immunotherapy. We are unaware of other reports suggesting prolonged survival using alum as an adjuvant.

In summary, in an effort to investigate how an IgE response against a tumor alters the course of the malignancy, an attempt to show that mice could make an IgE response against a tumor was undertaken. Extensive work concerned the lymphoma, L5178Y, and a lesser portion of study

was devoted to the melanoma, B16. IgE responses were not consistently demonstrated despite the prolonged survivals, indicating an apparent immune response, and the use of adjuvants, B. pertussis and alum, which are known to enhance IgE production, Clarification of methods in a report describing an IgE response to a tumor was obtained. This information along with the knowledge gained in the unsuccessful trials should enable this work to be successfully completed in the future.

(B) IgE Responses to Ovalbumin
in Tumor-Bearing Animals

Several reports have stated that individuals with neoplastic disease have depressed circulating levels of serum IgE.[9,13,102] If correct, these reports leave unanaswered the question of cause or effect. Has a low level of IgE predisposed to the malignancy or did the tumor result in depressed IgE? Only a very large prespective study would really answer these questions, but we attempted to gain some clues by studying IgE responses in mice bearing tumors and examining the effect of tumor inoculation on pre-existing IgE levels in mice. We are especially interested in reports of increased IgE in patients with Hodgkin's Disease.[9,198,231] As this is usually attributed to decreased T-cell function in what is thought by many to be a T-cell malignancy,[3] we studied IgE responses in mice bearing a theta positive lymphoma, L5178Y. The theta antigen is found on cells of thymic origin. We also assessed the effect of a melanoma on IgE responsiveness.

Methods:

Animals- Mice given living tumor cells were C57Bl/6J mice for

the melanoma studies or AKD2F1/J mice for the lymphoma studies. Both were purchased from Jackson laboratories, Bar Harbor, Maine. PCA reactions were titered in Swiss mice from the Sterling Hall of Medicine colony (2 hour IgG1 reactions) or in Sprague-Dawley rats (IgE reactions) from either Camm Research (Wayne, N.J.) or Charles River Labs (Wilmington, Mass.).

Tumors- Either the L5178Y lymphoma or the B16 melanoma were used as previously described. While the lymphoma was usually transferred from an in vivo cell line, one group of experiments made use of a transfer from an in vitro line also maintained by Dr. Robert Cappizzi.

Irradiation- Cells were irradiated with 3000 rads as previously described. Irradiated cells were used only in Experiment I.

Antigen- Ovalbumin (2X recrystallized, Worthington Biochemical, Freehold, N.J.) was given i.p. in saline at a dose of 1 ug with approximately 1 mg of alum, synthesized as previously described. Total injected volume was 0.1 ml.

PCA Reactions- These were run as previously described with passively sensitized mice or rats given i.v. injections of saline plus 0.7 % OA and 0.4% Evans blue two hours after skin sensitization. Mice were skin sensitized with 0.03 ml of diluted serum while 0.10 ml was used in the rats.

Results:

Experiments were designed to test if the transplanatation of tumor would affect the IgE response to ovalbumin. Experiments I through III involve the lymphoma L5178Y. In experiment I, tumor was given to mice that had a pre-existing, high titer of anti-OA antibodies. In experiment

II, the tumor was given just prior to the booster antigen. And in experiment III, tumor was transplanted just before or after the initial exposure to antigen. Experiments IV and V are similar except that they involve the melanoma B16. In the fourth, animals with a well established tumor were exposed to OA for the first time in the fifth, animals were given the tumor after a high, boosted antibody titer had been established. The data are summarized in the five accompanying tables (VI through X), with the data from Table VI also represented graphically in Figures II and III. Essentially animals bearing tumors were able to make IgE and IgG1 responses comparable to controls and once an animal had been immunized, transplantation of a tumor did not greatly reduce the antibody response, although a tendency toward depression of the antibody response was seen in some instances.

A few specific comments about each of the experiments are in order. In the first, (Table VI), high PCA titers were established by immunizing mice with OA and boosting them 28 days later. Several days after the booster dose, tumor was transferred either in a dose of 10^5 or 10^2 cells with one group receiving 10^5 cells that had been irradiated and one group receiving only saline. The graphs (Figures II and III) pictorialize that all four groups were within a two fold range of IgE and IgG1 concentrations at the time of tumor transplantation. The mice that received 10^5 cells had all died within 10 days. Irradiation only slightly delayed the death so that no animals in this group were living at day 15 after tumor transfer. All mice that received NaCl or 10^2 cells survived.

Effect of L5178Y on ongoing, boosted Antibody Response

Table VI Experiment I

# of Animals	Strain	Tumor Dose Cell #	Day Antigen Given	IgE Titer-Day* 7	12	17	22	27	IgG_1 Titer-Day* 7	12	17	22	27
6	AKD	.1cc NaCl	-35,-7	640	320	160	160	160	1280	1280	1280	2560	1280
6	AKD	10^5 l.p.	-35,-7	640	160	80			640	1280	320		
6	AKD	10^2 l.p.	-35,-7	640	320	160	80	80	640	1280	640	1280	1280
6	AKD	10^5 l.p. irradiated	-35,-7	320	80	40	40*		1280	1280	640	640**	

*Number of day is relative to last exposure to antigen
Serum from Day 7 was collected prior to transfer of tumor
**Serum was collected on Day 21 as animals were moribund at the time and all dead by Day 22
Blank spaces in table indicate all animals were dead by that bleeding date

FIGURE II

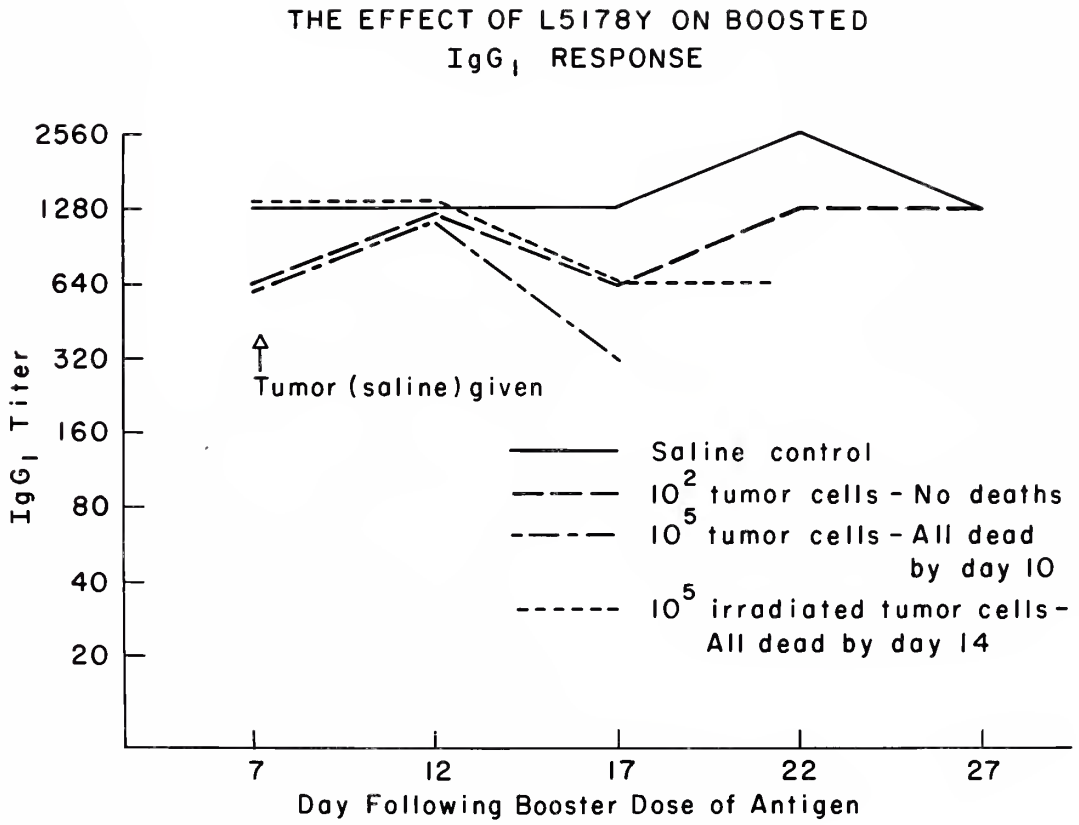
THE EFFECT OF L5178Y ON BOOSTED
IgG1 RESPONSE
2560
1280
640
320
160
80
40
20
IgG1 Titer
Tumor (saline) given
Saline control
10^2 tumor cells - No deaths
10^5 tumor cells - All dead by day 10
10^5 irradiated tumor cells - All dead by day 14
7
12
17
22
27
Day Following Booster Dose of Antigen

FIGURE III

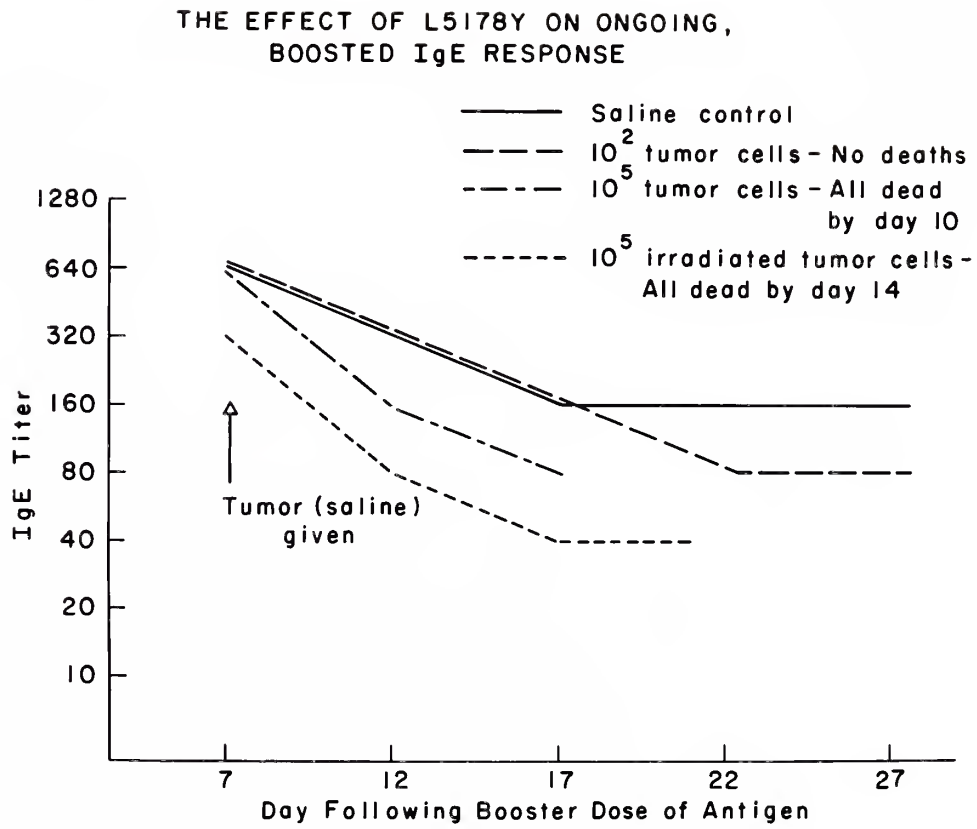
THE EFFECT OF L5178Y ON ONGOING, BOOSTED IgE RESPONSE
Saline control
10^2 tumor cells - No deaths
10^5 tumor cells - All dead by day 10
10^5 irradiated tumor cells - All dead by day 14
IgE Titer
1280
640
320
160
80
40
20
10
Tumor (saline) given
7
12
17
22
27
Day Following Booster Dose of Antigen

FIGURE III

As can be seen, the IgE levels did tend to drop faster and to lower levels in those mice dying from tumor, but as two fold differences were obtained in identically treated groups prior to tumor transplantation, the four fold differences seen after tumor implantation are suggestive but may not be significant. IgG1 levels tended to decline even less as compared to controls.

In experiment II (Table VII), antigen was given and then tumor was transferred just prior to the booster dose of antigen. The mice receiving tumor were dead or grossly ascitic fourteen days after tumor transfer. While the IgE levels do not differ significantly between the two groups, a marked decline in IgG1 levels is noted in the tumor-bearing mice just prior to death.

In experiment III (Table VIII), responses to the primary exposure to antigen were assessed, that is, no booster doses of OA were given. Tumor that had been maintained in vitro was injected either two days after antigen, the same day, or three days before. One group received the tumor s.c., a route of injection that permits slightly longer survival, and then was not given antigen until 12 days later when the tumor had been well established. One group was given tumor cells that had been frozen several months. As can be seen from the chart, all groups except the one receiving frozen cells produced IgE and IgG1 responses varying at most by a factor of two when compared with the controls that had received no tumor. The large majority of these animals died within 18 days of exposure to tumor with one animal surviving completely and one surviving to 22 days. If an animal did not appear ascitic when others in

Transfer of L5178Y Prior to Secondary Exposure to Antigen

Table VII Experiment II

# of Animals	Strain	Tumor Dose # of cells	Day Antigen Given Relative to tumor transfer	IgE titer		IgG_1 titer	
				*Day 5	*Day 11	*Day 5	*Day 11
3	AKD	1.2×10^5 l.p.	-24, + 3	320	160	160	160
3	AKD	None	-24, + 3	320	160	320	1280

*Number of day is relative to last exposure to antigen

Effect of L5178Y on primary exposure to Antigen

Table VIII Experiment III

# of Animals	Strain	Tumor Dose # of cells	Day Ag Given Relative to Tumor transfer	IgE titer *Day 12	IgE titer *Day 17	IgG_1 titer *Day 12	IgG_1 titer *Day 17
5	AKD	2×10^4 l.p.	0	20		5**	
4	AKD	2×10^4 l.p. Frozen	0	5		0	
5	AKD	2×10^4 l.p.	-2	80	10	10	0
5	AKD	2×10^4 l.p.	+3	80		10	
5	AKD	2×10^4 l.p.	+12	40		10	
4	AKD	None	0	40	10	5**	5

*Number of day is relative to last exposure to antigen
**Only two animals tested; dilution positive in 1 of 2

the group were clearly moribund, it was not bled. This was true of the one animal that survived the course of the experiment. Animals that received s.c. tumor died at about thirty days after inoculation and animals that had received frozen tumor cells also tended to die by this date. The death of those animals receiving frozen cells and their apparent lack of an IgE response will be commented upon in the discussion.

Experiments IV and V involved the melanoma B16. In the fourth (Table IX), we were attempting to confirm some preliminary data that suggested that mice bearing this tumor might be able to make a greater IgE response than controls. In this preliminary trial, a group of three mice had been given B16 plus a alum and three weeks later were immunized with OA plus alum. When they were bled one week later, their serum had an IgE titer of 1:20 while controls that had just received a comparable OA injection had no detectable titer. Neither group had detectable IgG1. Experiment IV could not confirm this finding as all groups had no measurable titers seven days after exposure to antigen. Thirteen days after antigen the groups still remained comparable, except that animals that had received s.c. saline did make a significantly higher IgG1 titer. No effort was made to repeat this and no explanation for it other than individual variation is readily apparent. While IgE concentrations for Day 13 were not tested at dilutions greater than 1:160, the qualitative response at high dilutions can give an approximation of the antibody titer and it was evident that these groups would have at most a two fold difference in IgE concentrations.

Effect of B16 on primary response to Antigen

Table IX Experiment IV

# of Animals	Strain	Tumor Dose # of Cells	Day Antigen Given Relative to tumor transfer	IgE titer *Day 7	IgE titer *Day 13	IgG_1 titer *Day 7	IgG_1 titer *Day 13
4	C57	9×10^4 S.C.	+20	0	All animals dead	0	All animals dead
4	C57	9×10^4 S.C. + Alum S.C.	+20	0	≥ 160	0	10
4	C57	Alum S.C.	+20	0	≥ 160	0	10
4	C57	Saline S.C.	+20	0	≥ 160	0	80

*Number of day is relative to last exposure to antigen

In the final experiment (Table X), mice that had been immunized with OA were given a booster dose 28 days later and a s.c. dose of tumor six days after that. On the day that the tumor was given, the controls had a two fold lower concentration of IgE and a four fold higher concentration of IgG1. These differences tended to disappear after one group was exposed to tumor, It should be noted that some of the titrations in this trial did not always establish the precise IgG1 concentration. The qualitative response at high dilutions suggested that IgG1 concentrations did not differ widely. As the primary purpose of the series of experiments was to examine IgE responses with IgG1 responses used as control data, precise titration of the IgG1 response was unnecessary if the IgE concentrations were within a two fold range.

Discussions:

The data lend little support to the idea that low levels of IgE arise from depressed IgE responsiveness during malignancy, but pre-existing antigen-specific IgE titers do tend to fall after a tumor is transplanted. However, in interpreting the data one should consider that: first, the method, may not be sensitive enough to detect decreased IgE responsiveness. For example, in experiment I four fold differences were noted between the IgE levels of tumor-bearing mice and the controls. These are suggestive differences, but as two fold differences occurred among groups of animals treated identically just prior to the tumor transfer, this four fold difference might be within experimental limits. IgG1 levels supposedly reflect levels of total antibody response.[230] Our data also suggest that tumor-bearing mice made a decreased IgG1 response, but again the limited sensitivity of the method makes such a

Effect of B16 on secondary response to antigen

Table X Experiment V

# of Animals	Strain	Tumor Dose # of cells	Day Antigen Given Relative to tumor transfer	I_gE titer			IgG_1 titer		
				6	*Day 13	20	6	*Day 13	20
4	C57	10^7 S.C.	-34,-6	160	40	10	40	$\geq$ 320	< 160
4	C57	None	-34,-6	80	40	20	160	$\geq$ 320	160

*Number of day is relative to last exposure to antigen
Titers from Day 6 were obtained from serum just prior to tumor transplant

conclusion difficult. Evidence in man supports the idea of a decreased antibody response in individuals with malignancy,[140] but reports of normal antibody responses have also been published.[209] Second, with PCA, RCA, one measures IgE titers for a specific antigen while reports in the literature have been concerned with total serum IgE. Several studies have shown generally normal or elevated levels of other immunoglobulins in most paitients with cancer,[259,260] but this may not be indicative of responsiveness to a specific antigen. Finally, to draw conclusions about human responses on the basis of animal work can be difficult. The most recently published study suggests that IgE levels are normal in patients with cancer.[231] Our data are not inconsistent with this concept.

The kinetics of the normal IgE response should be compared to the IgG1 response. IgE levels declined steadily after the booster dose of antigen, while the level of IgG1 tended to maintain itself or even rise slightly. The data suggest a more marked decline in IgE than IgG1. This might be explained in part by the much shorter half life of IgE,[234] at least in human studies, so that decreased immunoglobulin production following antigen exposure would become evident sooner for IgE than IgG1.

As mentioned in the literature review, Hodgkin's disease is unique among malignancies in that IgE levels are clearly elevated. Debate exists as to whether Hodgkin's disease is a T-cell or B-cell malignancy. The traditional view was that Hodgkin's disease must be a T-cell malignancy because T-cell function both as judged in vivo[39] and in vitro is depressed.[3,68] Recent evidence, however, suggests that T-cells in

Hodgkin's disease may be quite normal, only depleted in peripheral blood.[152] Some claim that the relative number of B-cells is also often diminished[68] and others suggest that the characteristic Reed-Sternberg cell has surface immunoglobulin and thus is of B-cell origin.[124] Perhaps the concept of T or B-cell malignancy is simply not relevant to this disease.

Regardless of which cell is primarily involved, unquestionably a depressed level of T-cell response often characterizes the disease. A wealth of excellent experimental data is now accumulating to elucidate the role of T-cells in controlling IgE responses[203,224,225] and the depressed T-cell function is one explanation of the elevated IgE.[198 231] Unfortunately, as alluded to previously, studying IgE responses in the mouse model of Hodgkin's disease, the SJL, is most difficult because at present they are thought to make poor IgE responses. The use of the lymphoma, L5178Y, seemed to offer a possible way to abrogate the T-cell control of IgE responses in mice. This lymphoma is theta positive;[169] that is, it bears an antigen marker attributed to lymphocytes of thymic origin. Speculation prior to the experiment was that a T-cell malignancy might be characterized by heightened IgE production. Most manipulations that destroy T-cell function including radiation,[175] anti-thymocyte serum,[176] and thymectomy,[175] generally predispose to increased IgE production. Increased IgE production clearly was not found in our experiments. Several explanations for this seem reasonable.

First, although T-cells may be affected by L5178Y, their function,

or at least their suppressor cell function, may be preserved. Second, as T-cells are known to exert both a helper[225] and a suppressor[223] effect over IgE production, perhaps affecting both equally allowed relatively normal IgE production. Third, the malignancy may have affected only a subset of all T-cells, leaving intact the subset vital for IgE response. Fourth, a tumor maintained in vivo or in vitro over a period of years may change its antigenic properties. Although we used the 'same' tumor that other investigators have found to be theta positive, only our own testing could verify this for our cell line. Finally, a transplanted malignancy might proliferate but leave the animal's indigenous lymphocytes functioning relatively normally.

Acute lymphocytic leukemia may be derived from T-cells.[112] In the few patients in whom it has been studied, IgE levels seem normal in this disease.[231] The tumor most clearly bearing T-cell determinants in man is the Sezary cell.[57] We are unaware of IgE levels having been measured in this disorder.

Finally, the decreased IgE levels in the mice given frozen tumor suggests a very speculative explanation. Viral infections, with measles being one example under study,[62,165] are known to affect immune responsiveness and remission of allergic symptoms has been described following measles vaccine.[265] Perhaps the diminished IgE response and subsequent death of these animals was due to an activation of a latent virus from the frozen, lysed cells.

(C) Serum levels of IgE in childhood neoplasia

If IgE confers a genetic selective advantage, this survival benefit must be present prior to child-bearing age. We wished, therefore, to measure IgE levels in children with neoplasias. As children normally have low levels of IgE which rise gradually into adolescence, we needed a radioimmunoassay that was particularly sensitive to low serum values. As explained in Part II, such an assay was not commercially available at the time of this study and so serum from children with a variety of tumors was collected and stored at -20°C for future investigation.

Some limitations on the measurement of total serum IgE in cases of neoplasia deserve comment. First, neoplasms represent a variety of entities, each with a theoretical variety of causes. Even limiting study to childhood neoplasia, we had collected samples which included acute lymphocytic leukemia, acute undifferentiated leukemia, lymphosarcoma, neuroblastoma, osteogenic sarcoma, and thyroid carcinoma. If total serum IgE is significant for any specific entity, this might be overlooked by considering all neoplasms together. Second, total serum IgE may be irrelevant as it might not reflect tumor-specific IgE. Normal levels of IgE vary widely so that a great deal of tumor-specific antibody might be present but not be reflected in total serum levels. Finally, finding abnormal levels of IgE would still leave the cause a mystery. Did low (or high) IgE predispose to malignancy or arise as a result?

(D) Tumor-Specific IgE Responses in Man

Almost a decade before the discovery of IgE as a unique class of immunoglobulins, a report was published indicating that IgE could be involved in the anti-tumor immune response of some individuals. The work of Grace,[73,74] Curtis[49] and co-workers showing that patients with cutaneous manifestations of malignancy had immediate wheal and flare reactions to their tumors established that IgE was involved, even though IgE was not known to be a separate antibody class at the time.

Our studies designed to demonstrate that some individual do make an IgE response against tumors had two purposes. First, we merely wished to verify the previous reports. Second, if we could readily develop a method to determine if a given individual was making an IgE response, then the implications of this response could be systematically evaluated. For example, what is the effect of IgE on the course and prognosis of the tumor? What characterizes the tumors and the individuals who tend to produce the IgE response? Does the presence of an IgE response suggest therapy?

The same method available to Grace and Curtis, namely the P-K reaction, was not available to us. Current ethical standards would not permit the transfer of solubilized human tumors to volunteers. Therefore, two alternative methods to establish the presence of an IgE response were evaluated, immunofluorescence and histamine release. The results with the former are described; the latter method is currently being investigated.

Most of the studies involve breast carcinoma because 1) five of Grace's patients had this disease,[74] 2) Black's data on skin windows shows that basophils are prognostically significant in breast cancer,[32,33] and 3) the disease is common enough so that tissue was readily available. We were unable to obtain tissue on any patient who had a cutaneous sign of malignancy.

Methods:

Tissue- All tissue was obtained either from surgical pathology or directly from the operating room usually within sixty minutes of its removal and often in less time. Tissue from 21 patients was obtained. Malignant tissue came from nine females with breast carcinoma and one with a breast sarcoma. Control tissue included sections from two reduction mammoplasties, biopsies from five cases of fibrocystic disease, breast tissue from a male with gynecomastia, and a spleen free of tumor from a patient with Hodgkin's disease. Tonsils and nasal polyps were obtained to provide positive controls. Immediately after its receipt, all tissues were frozen in liquid nitrogen and then stored at -90°C. Within a few weeks of its surgical removal, tissues were allowed to come to a temperature of -20°C and sectioned at a thickness of four to six microns with a cryostat. These sections were placed on microscope slides and stored at -90° C in sealed containers until ready for use, usually within a week or two. On one occasion, tissue from a patient with fibrocysiic disease was obtained directly from surgical pathology and immunofluorescence was performed without the intervening storage of tissue.

Antisera- Commercial antisera to human immunoglobulin G and E prepared in goats were purchased from Meloy Laboratories, Springfield, Va. These antisera were fluoresceinated with a fluorescein to protein ratio of 3.3 ug/mg for the anti-IgE and 6.2 ug/mg for the anti-IgG. Fluoresceinated antisera to human IgA and to rat immunoglobulin produced in goats were given to us by Dr. Michael Kashgarian. These had been purchased from Hyland Laboratories. All antisera were diluted with saline at a ratio of 1:5 except for the anti-IgG which was diluted 1:10.

Sera- Serum from the respective patients was obtained from the blood bank. Serum was diluted 1:10 with saline before use.

Immunofluorescence- Both direct and indirect immunofluorescence studies were usually performed. For indirect immunofluorescence one step in the procedure must involve incubation of the tissue with serum prior to the incubation with antiserum. For direct immunofluorescence this step is omitted. The direct technique detects antibody on the surface of the cell, while the indirect procedure looks for antibody in the serum that has become attached to cellular antigen

Slides were taken from the freezer at -90° C and allowed to come to room temperature before removing them from their sealed containers. Slides were then washed with agitation in phosphate buffered saline (PBS) for ten minutes. Slides were dried and placed over a moistened piece of filter paper. The tissue was then covered with the appropriate serum or antiserum. After a thirty minute incubation at 37° C slides were washed twice for ten minute periods in PBS while being shaken on a

platform. For direct immunofluorescence, slides were next dried, the tissue covered with a drop of phosphate buffered glycerine, and a coverslip put in place. For indirect studies, slides were subjected to a second incubation at 37°C, this time with appropriate antisera, and then two more ten minute washes were performed prior to being covered. Slides were viewed within hours of completion of the staining with a Zeiss microscope equipped with ultraviolet light.

Histology- Hematoxylin and eosin (H&E) sections and phase contrast microscopy were performed with some of the sections in order to localize the areas of fluorescence.

Results:

Initial studies involved only the Meloy antisera to IgG and IgE. These studies indicated that the sections from four of six patients with malignancy stained with both of the fluoresceinated antisera after direct and indirect immunofluorescence. Tissue from the five patients with fibrocystic disease and the two patients with reduction mammoplasties stained with anti-IgG consistently, but never significantly with anti-IgE. Both benign and malignant tissue stained occasionally with anti-IgE in areas of vascular smooth muscle. Such staining was not considered positive.

With the help of Dr. Michael Kashgarian, H & E stains of the slides were prepared. Examining the slides under light and phase contrast microscopy verified that the immunofluorescence in the positive specimens was predominantly over the area of malignant cells. However, when the control of fluoresceinated anti-rat immunoglobulin was added to the

procedure, this antiserum too stained the areas with malignant tissue in a pattern identical to that obtained with anti-IgE.

Some of the tissues collected including the sarcomatous breast and the spleen from the paitent with Hodgkin's disease have not been sectioned but remain in storage in -90°C. Their ultimate use will depend upon the progress of further immunofluorescence studies.

Discussion:

Immunofluorescence as a technique for demonstrating a humoral immune reaction to a tumor was first described by Klein et al.[118] in patients with Burkitt's lymphoma. While immunofluorescence has been used to locate IgE in such diverse studies as those on nasal polyps,[18] the thyroid in Graves' disease,[239] the kidney in the nephrotic syndrome,[70] [133] and peripheral blood lymphocytes in a variety of disease states,[5,67, 120,155,181] we are unaware of any previous study that has used immunofluorescence to investigate the role of IgE in response to a solid tumor.

Our initial results were extremely encouraging, but performance of adequate controls indicated that this enthusiasm was premature. The artifactual staining of tumor tissue may be due to a number of factors including necrosis of the cells despite our diligence in freezing the tissue as rapidly as possible. The malignant cells may be more sensitive to the experimental conditions and thus they attracted the anti-IgE while the benign tissue samples may have had less necrosis. Breast tissue does have significant local immunoglobulin production[193,194] and this might explain the staining for anti-IgG in all tissues, benign and malignant. Another possibility is that these malignant cells are especi-

ally cytophilic for proteins.

Immunofluorescence studies not involving IgE have been performed with breast carcinoma.[188] However, these studies involved tumor cells that have been grown in culture. With such a system, one can more easily control the staining procedure and artifact by using a set number of cells for each trial. As human tumors including breast carcinoma[252] tend to have common antigenic determinants, a single tumor growing in culture could be used to test sera of numerous patients with the technique of indirect immunofluorescence. Such testing would assume that the common antigen(s) is the one recognized by IgE. While breast carcinoma is not considered one of the easier tumors to grow in culture, cells obtained from pleural effusions secondary to the tumor do grow fairly readily in vitro.

We are currently approaching the problem of demonstrating that some individuals produce tumor-specific IgE with the use of a histamine release assay. Basophils in high concentration can be found among the leukocytes from blood sedimented with dextran or Ficoll-Hypaque.[264] If a paitent has produced IgE specific for his tumor, this IgE should have passively sensitized his own basophils. Adding solubilized tumor antigen to these leukocytes should cause the release of histamine as the cell degranulates.

If human tumors do cause the release of histamine, the implications may relate to both pathogenesis and therapeutics. Histamine or other vasoactive substances released by IgE may be implicated in the cutaneous signs of some tumors. And substances that block or neutralize the pro-

ducts of an IgE-antigen reaction would be logical pharmacologic agents to evaluate.

Paradoxes in evaluating the putative IgE response to tumors are plentiful. The data on CBH suggests that the basophil can play a role in tumor rejection, while the present understanding of histamine's effect on the immune response suggests that it should hamper tumor rejection. Work with adjuvants like B. pertussis implies to some that a tumor-specific IgE response is beneficial, but reports on cutaneous manifestations of malignancy suggest that IgE may be the culprit. IgE is elevated in Hodgkin's disease presumably secondary to depressed T-cell function, but lowered T-cell function is found often in malignancy and IgE levels are either normal or low. Is atopy related to the tendency to make a tumor-specific IgE response? Do skin signs appear in the absence of an IgE response? Does an IgE response occur in the absence of skin signs? Why should IgE be related to dermatologic disease? A histamine release assay is a reliable, reproducible technique that should greatly facilitate the answering of these questions.

SUMMARY

The possible role of IgE in response to neoplasia is reviewed with speculations offered about the possible implications of this response. Experimental efforts were divided bwtween basic research on IgE and studies directly involving tumors. Of the basic studies, A) the use of a solid phase radioimmunoassay (RIA) was perfected; B) evaluation of diurnal rhythms awaits commercial availability or a more sensitive, reproducible assay; and C) preliminary work on the ontogenetics of IgE suggests that its synthesis may be independent of the prior maturation of IgM bearing cells. Of the tumor-related studies, A) the demonstration in animals of IgE specific for tumor antigen has progressed so that its accomplishment should be attainable; B) the studies on IgE responses to ovalbumin in tumor-bearing mice suggest that the tumor-bearing animals make antibody responses nearly comparable to controls even with the transplantion of a theta positive lymphoma; C) the work on IgE levels in childhood malignancies must await the aforementioned RIA; and D) studies now in progress are attempting to demonstrate IgE responses to tumors in human subjects with the use of immunofluorescence and a histamine release assay.

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